



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : C07K 16/00, C12N 15/12, 15/13, 15/63, G01N 33/53, A61K 38/17, 39/395		A1	(11) International Publication Number: <b>WO 98/02462</b> (43) International Publication Date: 22 January 1998 (22.01.98)		
(21) International Application Number: PCT/EP97/03792		(81) Designated States: CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).			
(22) International Filing Date: 16 July 1997 (16.07.97)					
(30) Priority Data: 96111441.0 16 July 1996 (16.07.96) EP (34) Countries for which the regional or international application was filed: DE et al.		Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>			
(71) Applicant (for all designated States except US): MORPHOSYS GESELLSCHAFT FÜR PROTEINOPTIMIERUNG MBH [DE/DE]; Am Klopferspitz 19, D-82152 Martinsried (DE).					
(71)(72) Applicant and Inventor: PLÜCKTHUN, Andreas [DE/CH]; Möhlistrasse 97, CH-8006 Zürich (CH).					
(72) Inventors; and					
(75) Inventors/Applicants (for US only): NIEBA, Lars [DE/CH]; Nordstrasse 304, CH-8037 Zürich (CH). HONEGGER, Annemarie [CH/CH]; Murwiesenstrasse 32, CH-8057 Zürich (CH).					
(74) Agent: VOSSIUS & PARTNER GBR; P.O. Box 86 07 67, D-81634 München (DE).					
(54) Title: IMMUNOGLOBULIN SUPERFAMILY DOMAINS AND FRAGMENTS WITH INCREASED SOLUBILITY					
(57) Abstract					
<p>The present invention relates to the modification of immunoglobulin superfamily (IgSF) domains, IgSF fragments and fusion proteins thereof, especially to the modification of antibody derivatives, so as to improve their solubility, and hence the yield, and ease of handling. The inventors have found that this can be achieved by making the region which comprises the interface with domains adjoined to said IgSF domain in a larger fragment or a full IgSF protein, and which becomes exposed in the IgSF domain, more hydrophilic by modification. The present invention describes DNA sequences encoding modified IgSF domains or fragments and fusion proteins thereof, vectors and hosts containing these DNA sequences, IgSF domains or fragments or fusion proteins obtainable by expressing said DNA sequences in suitable expression systems, and a method for modifying IgSF domains, so as to improve their solubility, expressibility and ease of handling.</p>					

BEST AVAILABLE COPY

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

## Immunoglobulin Superfamily Domains and Fragments with Increased Solubility

### Field of the Invention

The present invention relates to the modification of immunoglobulin superfamily (IgSF) domains and derivatives thereof so as to increase their solubility, and hence the yield, and ease of handling.

### Background to the Invention

Small antibody fragments show exciting promise for use as therapeutic agents, diagnostic reagents, and for biochemical research. Thus, they are needed in large amounts, and the expression of antibody fragments, e.g. Fv, single-chain Fv (scFv), or Fab in the periplasm of *E. coli* (Skerra & Plückthun 1988; Better et al., 1988) is now used routinely in many laboratories. Expression yields vary widely, however, especially in the case of scFvs. While some fragments yield up to several mg of functional, soluble protein per litre and OD of culture broth in shake flask culture (Carter et al., 1992; Plückthun et al. 1996), other fragments may almost exclusively lead to insoluble material, often found in so-called inclusion bodies. Functional protein may be obtained from the latter in modest yields by a laborious and time-consuming refolding process. The factors influencing antibody expression levels are still only poorly understood. Folding efficiency and stability of the antibody fragments, protease lability and toxicity of the expressed proteins to the host cells often severely limit actual production levels, and several attempts have been tried to increase expression yields. For example, Knappik & Plückthun (1995) have identified key residues in the antibody framework which influence expression yields dramatically. Similarly, Ulrich et al. (1995) found that point mutations in the CDRs can increase the yields in periplasmic antibody fragment expression. Nevertheless, these strategies are only applicable to a few antibodies.

The observations by Knappik & Plückthun (1995) indicate that optimizing those parts of the antibody fragment which are not directly involved in antigen recognition can significantly

improve folding properties and production yields of recombinant Fv and scFv constructs. The causes for the improved expression behavior lie in the decreased aggregation behavior of these molecules. For other molecules, fragment stability and protease resistance may also be affected. The understanding of how specific sequence modifications change these properties is still very limited and currently under active investigation.

Difficulties in expressing and manipulating protein domains may arise because amino acids which are normally buried within the protein structure become exposed when only a portion of the whole molecule is expressed. Aggregation may occur through interaction of newly solvent-exposed hydrophobic residues originally forming the contact regions between adjacent domains. Leistler and Perham (1994) could show that a certain domain of glutathione reductase may be expressed separately from its neighboring domains, but the protein showed non-specific association *in vitro* forming multimeric protein species. The introduction of hydrophilic residues instead of exposed hydrophobic amino acids could decrease this aggregation tendency and thus stabilize this isolated domain. Both wild type and modified domains were exclusively found in inclusion bodies and had to be refolded. Although *in vitro* experiments contributed a lot to define various intermolecular interactions, which drive folding processes, they are only of limited value in predicting the folding behaviour of different polypeptide chains *in vivo* (Gething & Sambrook, 1992). Thus, Leistler and Perham do not teach or suggest how to increase expression yields of soluble protein domains.

In the case of antibodies, two chains comprising several domains dimerize, each domain consisting of a  $\beta$ -barrel whose two  $\beta$ -sheets are held together by a disulphide bond, forming the so-called immunoglobulin fold. Two domains, one variable domain (VL) and one constant domain (CL) are adjacent along the longitudinal axis in the light chain (VL-CL), and four domains, one variable domain (VH) and three constant domain (CH1 to CH3) are adjacent along the longitudinal axis in the heavy chain (VH-CH1-CH2-CH3). In the dimer formed by chains a and b, two such domains associate laterally: VL<sub>a</sub> with VH<sub>a</sub>, CL<sub>a</sub> with CH1<sub>a</sub>, VL<sub>b</sub> with VH<sub>b</sub>, CL<sub>b</sub> with CH1<sub>b</sub>, CH2<sub>a</sub> with CH2<sub>b</sub> and CH3<sub>a</sub> with CH3<sub>b</sub>. In WO 92/01787 (Johnson et al., 1992), it is taught that isolated single domains, e.g. VH, can be modified in the former VL/VH interface region by exchanging hydrophobic residues by hydrophilic ones without changing the specificity of the parent domain. The rationale for WO 92/01787 was the

assumption that exposed hydrophobic residues might lead to non-specific binding, interaction with surfaces and decreased stability. Data for increase in binding specificity was given, but increase in expression level was not shown. Furthermore, WO 92/01787 would not be applicable to any antibody fragment containing the complete antigen binding site, as it must contain VI and VII.

In the case of T cell receptors, two chains ( $\alpha$  and  $\beta$ ) dimerize, each consisting of a variable (V) and a constant (C) domain with the immunoglobulin fold, and one transmembrane domain. In each chain, the variable and constant domains are adjacent along the longitudinal axis in the chains (V $\alpha$ -C $\alpha$ ; V $\beta$ -C $\beta$ ) and associate laterally with the corresponding domains of the second chain (V $\alpha$ -V $\beta$ ; C $\alpha$ -C $\beta$ ).

Various other molecules of the immunoglobulin superfamily, such as CD2, CD4, CD16, CD22, comprise only one chain, wherein two or more domains (variable and/or constant) with the immunoglobulin fold are adjacent along the longitudinal axis in the chains.

The present inventors have found that expression problems are largely associated with a part of the molecule that has hitherto not been regarded relevant for expression studies and which comprises the interface between adjacent domains within an immunoglobulin chain. This surprising finding forms the basis of the present invention, which provides a general solution to the problems associated with production of domains or fragments of the immunoglobulin superfamily (IgSF), especially antibody fragments, which exhibit poor solubility or reduced levels of expression.

### **Detailed Description of the Invention**

In addition to lateral interactions between domains of different chains described above, there are well documented contacts between adjacent domains within individual chains along the longitudinal axis. For example, in the case of an antibody (Lesk & Chothia, 1988), the "bottom" of VL makes contact with the "top" of CL, and, in a similar manner there are contacts between VII and CH1. The contacts at these inter-domain interfaces are probably essential for the compact arrangement of the Fab fragment, and, as is typical for such contacts, are at least partially hydrophobic in nature (Lesk & Chothia, 1988).

The basis of the present invention is the surprising finding that the solubility (and hence the yield) of antibody fragments comprising at least one domain can be dramatically increased by decreasing the hydrophobicity of former interfaces at the "end" of said domain, where it would normally adjoin a second domain within a chain in a larger antibody fragment or full antibody. This is surprising and could not have been predicted from the prior art (WO 92/01787), because the size of the longitudinal interface, for example, in a scFv fragment, is much smaller than that between VH and VL, and therefore, the amino acids which make up the interfaces between VH and CH1 or between VL and CL in a Fab fragment represent a much smaller proportion of the total surface area of the scFv molecule, and would accordingly be expected to play less of a role in determining the physical properties of the molecule.

The present invention has the additional advantage that because the alterations effected in the molecules that lead to said decreased hydrophobicity of former interfaces are located at the most distant part of the domain from the CDRs, applying the invention is unlikely to have a deleterious effect on the binding properties of the molecule. This is not the case in WO 92/01787, where at least one modification is close to the CDRs and may therefore be expected to have an impact on antigen binding. Furthermore, WO 92/01787 cannot be applied to VL/VH heterodimers, as explained above.

The present invention relates to a modified immunoglobulin superfamily (IgSF) domain or fragment which differs from a parent IgSF domain or fragment in that the region which comprised or would comprise the interface with a second domain adjoined to said parent IgSF domain or fragment within the protein chain of a larger IgSF fragment or a full IgSF protein, and which is exposed in said parent IgSF domain or fragment in the absence of said second domain, is made more hydrophilic by modification.

In the context of the present invention, the term immunoglobulin superfamily (IgSF) domain refers to those parts of members of the immunoglobulin superfamily which are characterized by the immunoglobulin fold, said superfamily comprising the immunoglobulins or antibodies, and various other proteins such as T-cell receptors or integrins. The term IgSF fragment refers to any portion of a member of the immunoglobulin superfamily, said portion comprising at least one IgSF domain. The term adjoining domain refers to a domain which is contiguous with a first domain. The term interface refers to a region of said first domain where interaction with the adjoining domain takes place. The terms hydrophobic and hydrophilic refer to a

physical property of amino acids, which can be estimated quantitatively: tabulated values of hydrophobicity for the twenty naturally-occurring amino acids are available (Nozaki & Tanford, 1971; Casari & Sippl, 1992; Rose & Wolfenden, 1993).

The residues to be modified can be identified in a number of ways. For example, in one way, the solvent accessibilities (Lee & Richards, 1971) of hydrophobic interface residues in said parent IgSF fragment compared to said larger IgSF fragment or full IgSF protein are calculated, with high accessibilities indicating highly exposed residues. In a second way, the number of van der Waals contacts of hydrophobic interface residues in said larger IgSF fragment or full IgSF protein is calculated. A large number for a residue of said parent domain indicates that it will be highly solvent-exposed in the absence of an adjoining domain. There are other ways of calculating or determining residues to be modified according to the present invention, and one of ordinary skill in the art will be able to identify and practice these ways.

By analyzing computer models of said parent IgSF fragment, interactions of said highly exposed residues within the fragment can be identified. Such interactions could stabilize the parent IgSF fragment. Residues, which interact closely with other hydrophobic residues and which can be identified by anyone of ordinary skill in the art, should not preferentially be mutated.

The modification referred to above may be effected in a number of ways which are well known to one skilled in the art. In a preferred embodiment, the modification is a substitution of one or more amino acids at the exposed interface, identified as described above, with amino acids which are more hydrophilic. Alternatively, one or more amino acids can be inserted in said interface, or one or more amino acids can be deleted from said interface, so as to increase its overall hydrophilicity. Furthermore, any combination of substitution, insertion and deletion can be effected to reduce the hydrophobicity of said interface. Also comprised by the present invention is the possibility that the substitution or insertion comprises amino acids with a relatively high hydrophobicity value, or that the deletion comprises amino acids with relatively low hydrophobicity value, as long as the overall hydrophilicity value is increased in said interface region. Modifications such as substitution, insertion and deletion can be effected using standard methods which are well known to practitioners skilled in the art. By way of example, the skilled artisan can use either site-directed or PCR-based mutagenesis (Ho et al., 1989; Kunkel et al., 1991; Trower, 1994; Viville, 1994), or total gene synthesis (Prodromou &

Pearl, 1992) to effect the necessary modification(s). In a further embodiment, the mutations may be obtained by random mutagenesis and screening of random mutants, using a suitable expression and screening system (see, for example, Stemmer, 1994; Crameri et al., 1996).

In a preferred embodiment, the amino acid(s) which replace(s) the more hydrophobic amino acids include Asn, Asp, Arg, Gln, Glu, Gly, His, Lys, Ser, and Thr. These are among the more hydrophilic of the 20 naturally-occurring amino acids, and have proven to be particularly effective in the application of the present invention. Said amino acids, alone or in combination, or in combination with other amino acids, can also be used to form the above mentioned insertion which makes the interface region more hydrophilic.

The parent IgSF domain or fragment referred to above can be one of several different types. In a preferred embodiment, said parent domain or fragment is derived from an antibody. In one embodiment, said parent antibody fragment comprises an Fv fragment. In this context, the term Fv fragment refers to a complex comprising the VL (variable light) and VH (variable heavy) portions of the antibody molecule. In a further embodiment, the parent antibody fragment may be a single-chain Fv fragment (scFv; Bird et al., 1988; Huston et al., 1988), in which the VL and VH chains are joined, in either a VL-VH, or VH-VL orientation, by a peptide linker. In yet a further embodiment, the parent antibody fragment may be an Fv fragment stabilized by an inter-domain disulphide bond. This is a structure which can be made by engineering into each chain a single cysteine residue, wherein said cysteine residues from two chains become linked through oxidation to form a disulphide (Glockshuber et al., 1990; Brinkmann et al., 1993).

In a most preferred embodiment, the interface region of the variable domains mentioned above comprises residues 9, 10, 12, 15, 39, 40, 41, 80, 81, 83, 103, 105, 106, 106A, 107, 108 for VL, and residues 9, 10, 11, 13, 14, 41, 42, 43, 84, 87, 89, 105, 108, 110, 112, 113 for VH according to the Kabat numbering system (Kabat et al., 1991). Said numbering system was established for the sequences of whole antibodies, but can be adapted correspondingly to describe the sequences of isolated antibody domains or antibody fragments, even in the case of scFv fragments, where VL and VH are connected via a peptide linker, and where the protein sequence from N- to C-terminus has to be numbered differently. This means that the Kabat numbering system is used in the present invention as a sequence description relative to the

existing data on antibody sequences, not as an absolute description of actual positions within the antibody fragment sequences of interest.

In a further embodiment, said parent antibody fragment comprises a Fab fragment. In this context, the term Fab refers to a complex comprising the VI-CL (variable and constant light) and VII-CH1 (variable and first constant heavy) portions of the antibody molecule, and the term interface region refers to a region in the first constant domain of the heavy chain (CH1) which is, or would be adjoined to, the CH2 domain in a larger antibody fragment or full antibody.

In a still further embodiment, said parent IgSF fragment is a fusion protein of any of said domains or fragments and another protein domain, derived from an antibody or any other protein or peptide. The advent of bacterial expression of antibody fragments has opened the way to the construction of proteins comprising fusions between antibody fragments and other molecules. A further embodiment of the present invention relates to such fusion proteins by providing for a DNA sequence which encodes both the modified IgSF domain or fragment, as described above, as well as an additional moiety. Particularly preferred are moieties which have a useful therapeutic function. For example, the additional moiety may be a toxin molecule which is able to kill cells (Vitetta et al., 1993). There are numerous examples of such toxins, well known those skilled in the art, such as the bacterial toxins Pseudomonas exotoxin A, and diphtheria toxin, as well as the plant toxins ricin, abrin, modeccin, saporin, and gelonin. By fusing such a toxin to an antibody fragment, the toxin can be targeted to, for example, diseased cells, and thereby have a beneficial therapeutic effect. Alternatively, the additional moiety may be a cytokine, such as IL-2 (Rosenberg & Lotze, 1986), which has a particular effect (in this case a T-cell proliferative effect) on a family of cells. In a further preferred embodiment, the additional moiety is at least part of a surface protein which may direct the fusion protein to the surface of an organism, for example, a cell or a phage, and thereby displays the IgSF partner. Preferably, the additional moiety is at least part of a coat protein of filamentous bacteriophages, most preferably of the genelll protein. In a further embodiment, the additional moiety may confer on its IgSF partner a means of detection and/or purification. For example, the fusion protein could comprise the modified IgSF domain or fragment and an enzyme commonly used for detection purposes, such as alkaline phosphatase (Blake et al., 1984). There are numerous other moieties which can be used as detection or

purification tags, which are well known to the practitioner skilled in the art. Particularly preferred are peptides comprising at least five histidine residues (Hochuli et al., 1988), which are able to bind to metal ions, and can therefore be used for the purification of the protein to which they are fused (Lindner et al., 1992). Also provided for by the invention are additional moieties such as the commonly used c-myc and FLAG tags (Hopp et al., 1988; Knappik & Plückthun, 1994).

By engineering one or more fused additional domains, IgSF domains or fragments can be assembled into larger molecules which also fall under the scope of the present invention. To the extent that the physical properties of the IgSF domain or fragment determine the characteristics of the assembly, the present invention provides a means of increasing the solubility of such larger molecules. For example, mini-antibodies (Pack, 1994) are dimers comprising two antibody fragments, each fused to a self-associating dimerization domain. Dimerization domains which are particularly preferred include those derived from a leucine zipper (Pack & Plückthun, 1992) or helix-turn-helix motif (Pack et al., 1993).

All of the above embodiments of the present invention can be effected using standard techniques of molecular biology known to anyone skilled in the art.

The compositions described above may have utility in any one of a number of settings. Particularly preferred are diagnostic and therapeutic compositions.

The present invention also provides methods for making the compositions and compounds comprised therein described above. Particularly preferred is a method comprising the following steps:

- i) analyzing the interface region of an IgSF domain for hydrophobic residues which are solvent-exposed using either a solvent-accessibility approach (Lee & Richards, 1971), analysis of van der Waals interactions in the interface region, or similar methods which are well known to one skilled in the art.
- ii) identifying one or more of the hydrophobic residues to be substituted by more hydrophilic residues, or one or more positions where hydrophilic residues or amino acid stretches enhancing the overall hydrophilicity of the interface region can be inserted into said interface region, or one or more positions where hydrophobic residues or amino acid

-9-

stretches enhancing the overall hydrophobicity of the interface region can be deleted from said interface region, or any combination of said substitutions, said insertions, and said deletions to give one or more mutants of said parent IgSF domain.

- iii) preparing DNA encoding mutants of said IgSF domain, characterized by the changes identified in ii), by using e.g. conventional mutagenesis or gene synthesis methods, said DNA being prepared either separately or as a mixture.
- iv) introducing said DNA or DNA mixture in a vector system suitable for expression of said mutants.
- v) introducing said vector system into suitable host cells and expressing said mutant or mixture of mutants.
- vi) identifying and characterizing mutants which are obtained in higher yield in soluble form, and
- vii) if necessary, repeating steps iii) to vi) to increase the hydrophilicity of said identified mutant or mutants further.

The host referred to above may be any of a number commonly used in the production of heterologous proteins, including but not limited to bacteria, such as *E. coli* (Ge et al. 1995), or *Bacillus subtilis* (Wu et al., 1993), fungi, such as yeasts (Horwitz et al., 1988; Ridder et al., 1995) or filamentous fungus (Nyyssönen et al., 1993), plant cells (Hiatt, 1990, Hiatt & Ma, 1993; Whitelam et al., 1994), insect cells (Potter et al., 1993; Ward et al., 1995), or mammalian cells (Trill et al., 1995).

The invention also relates to a method for the production of an IgSF domain or fragment of the invention comprising culturing a host cell of the invention and isolating said domain or fragment.

The invention is now demonstrated by the following examples, which are presented for illustration only and are not intended to limit the scope of the invention.

**Examples****i) Abbreviations**

Abbreviations: CDR: complementarity determining region; dsFv: disulfide-linked Fv fragment; IMAC: immobilized metal ion affinity chromatography; IPTG: isopropyl- $\beta$ -D-thiogalactopyranoside; i/s: ratio insoluble/soluble; H(X): heavy chain residue number X; L(X): light chain residue number X; NTA: nitrilo-triacetic acid; OD<sub>550</sub>: optical density at 550 nm; PDB: protein database; scFv: single-chain Fv fragment; SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis; v/c: variable/constant; wt: wild type

**ii) Material and Methods****(a) Calculation of solvent accessibility**

Solvent accessible surface areas for 30 non-redundant Fab fragments and the Fv fragments derived from these by deleting the constant domain coordinates from the PDB file were calculated using the latest version, as of March 1996, of the program NACCESS (<http://www.biochem.ucl.ac.uk/~roman/naccess/naccess>) based on the algorithm described by Lee & Richards (1971).

**(b) scFv gene synthesis**

The single-chain Fv fragment (scFv) in the orientation V<sub>L</sub>-linker-V<sub>H</sub> of the antibody 4-4-20 (Bedzyk et al., 1990) was obtained by gene synthesis (Prodromou and Pearl, 1992). The V<sub>L</sub> domain carries a three-amino acid long FLAG tag (Knappik and Plückthun, 1994). We have used two different linkers with a length of 15 (Gly4Ser)<sub>3</sub> and 30 amino acids (Gly4Ser)<sub>6</sub>, respectively. The gene so obtained was cloned into a derivative of the vector pIG6 (Ge et al., 1995). The mutant antibody fragments were constructed by site-directed mutagenesis (Kunkel et al., 1987) using single-stranded DNA and up to three oligonucleotides per reaction.

## (c) Expression

Growth curves were obtained as follows: 20 ml of 2xYT medium containing 100 µg/ml ampicillin and 25 µg/ml streptomycin were inoculated with 250 µl of an overnight culture of *E. coli* JM83 harboring the plasmid encoding the respective antibody fragment and incubated at 24.5°C until an OD<sub>550</sub> of 0.5 was reached. IPTG (Biomol Feinchemikalien GmbH) was added to a final concentration of 1 mM and incubation was continued for 3 hours. The OD was measured every hour, as was the β-lactamase activity in the culture supernatant to quantify the degree of cell leakiness. Three hours after induction an aliquot of the culture was removed and the cells were lysed exactly as described by Knappik and Plückthun (1995). The β-lactamase activity was measured in the supernatant, in the insoluble and in the soluble fraction. The fractions were assayed for antibody fragments by reducing SDS-PAGE, with the samples normalized to OD and β-lactamase activity to account for possible plasmid loss as well as for cell leakiness. The gels were blotted and immunostained using the FLAG antibody M1 (Prickett et al., 1989) as the first antibody, an Fc-specific anti-mouse antiserum conjugated to horseradish peroxidase (Pierce) as the second antibody, using a chemoluminescent detection assay described elsewhere (Ge et al., 1995).

## (d) Purification

Mutant scFv fragments were purified by a two-column procedure. After French press lysis of the cells, the raw *E. coli* extract was first purified by IMAC (Ni-NTA superflow, Qiagen) (20 mM HEPES, 500 mM NaCl, pH 6.9; step gradient of imidazole 10, 50 and 200 mM) (Lindner et al., 1992) and, after dialyzing the IMAC eluate against 20 mM MES pH 6.0, finally purified by cation exchange chromatography (S-Sepharose fast flow column, Pharmacia) (20 mM MES, pH 6.0; salt gradient 0-500 mM NaCl). Purity was controlled by Coomassie stained SDS-PAGE. The functionality of the scFv was tested by competition ELISA.

Because of its very poor solubility in the periplasmic system, the wt 4-4-20 was expressed as cytoplasmic inclusion bodies in the T7-based system (Studier & Moffatt, 1986; Ge et al., 1995). The refolding procedure was carried out as described elsewhere (Ge et al., 1995). For

purification, the refolding solution (2 l) was loaded over 10 h without prior dialysis onto a fluorescein affinity column, followed by a washing step with 20 mM HEPES, 150 mM NaCl, pH 7.5. Two column volumes of 1 mM fluorescein (sodium salt, Sigma Chemicals Co.) pH 7.5 were used to elute all functional scFv fragment. Extensive dialysis (7 days with 12 buffer changes) was necessary to remove all fluorescein. All purified scFv fragments were tested in gel filtration (Superose-12 column, Pharmacia SMART-System, 20 mM HEPES, 150 mM NaCl, pH 7.5).

(e)  $K_D$  determination by fluorescence titration

The concentrations of the proteins were determined photometrically using an extinction coefficient calculated according to Gill and von Hippel (1989). Fluorescence titration experiments were carried out by taking advantage of the intensive fluorescence of fluorescein. Two ml of 20 mM HEPES, 150 mM NaCl, pH 7.5 containing 10 or 20 nM fluorescein were placed in a cuvette with integrated stirrer. The excitation wavelength was 485 nm, emission spectra were recorded from 490 to 530 nm. Purified scFv (in 20 mM HEPES, 150 mM NaCl, pH 7.5) was added in 5 to 100  $\mu$ l aliquots, and after a 3 min equilibration time a spectrum was recorded. All spectra were recorded at 20°C. The maximum of emission at 510 nM was used for determining the degree of complexation of scFv to fluorescein, seen as quenching as a function of the concentration of the antibody fragment. The  $K_D$  value was determined by Scatchard analysis.

(f) Equilibrium denaturation measurement

Equilibrium denaturation curves were obtained by denaturation of 0.2  $\mu$ M protein in HEPES buffered saline (HBS) buffer (20 mM HEPES, 150 mM NaCl, 1 mM EDTA, pH 7.5) and increasing amounts of urea (1.0-7.5 M; 20 mM HEPES, 150 mM NaCl, pH 7.4; 0.25 M steps) in a total volume of 1.7 ml. After incubating the samples at 10°C for 12 hours and an additional 3 hours at 20°C prior to measurements, the fluorescence spectra were recorded at 20°C from 320-360 nm with an excitation wavelength of 280 nm. The emission wavelength of the fluorescence peak shifted from 341 to 347 nm during denaturation and was used for determining the fraction of unfolded molecules. Curves were fitted according to Pace (1990).

## (g) Thermal denaturation

For measuring the thermal denaturation rates, purified scFv was dissolved in 2 ml HBS buffer to a final concentration of 0.5  $\mu$ M. The aggregation was followed for 2.5 h at 40°C and at 44°C by light scattering at 400 nm.

## iii) Results

## (a) Comparison of known antibody sequences

Compared to other domain/domain interfaces in proteins, the interface between immunoglobulin variable and constant domains is not very tightly packed. A comparison of 30 non-redundant Fab structures in the PDB database showed that between the light chain variable and constant domain an area of  $410 \pm 90 \text{ \AA}^2$  per domain is buried, while the heavy chain variable and constant domains interact over an area of  $710 \pm 180 \text{ \AA}^2$ . Some, but not all of the interface residues are hydrophobic, predominantly aliphatic. Generally, sequence conservation of the residues contributing to the v/c domain interface is not particularly high. Still, the v/c domain interface shows up as a marked hydrophobic patch on the surface of an Fv fragment (Fig. 1).

Solvent accessible surface areas for 30 non-redundant Fab fragments and their corresponding Fv fragments (derived from the Fab fragment by deleting the constant domain coordinates from the PDB file) were calculated using the program NACCESS (Lee & Richards, 1971). Residues participating in the v/c domain interface were identified by comparing the solvent-accessible surface area of each amino acid side chain in the context of an Fv fragment to its accessible surface in the context of an Fab fragment. Figure 2 shows a plot of the relative change in side chain accessibility upon deletion of the constant domains as a function of sequence position. Residues which show a significant reduction of side chain accessibility are also highlighted in the sequence alignment. To assess sequence variability in the positions identified in Figure 2, the variable domain sequences collected in the Kabat database (status March 1996) were analyzed (Table 1). Of the 15 interface residues identified in the VL

-14-

domain of the antibody 4-4-20 (Fig. 1 and Table 1). L9(Leu), L12(pro), L15(Leu), L40(pro), L83(Leu), and L106(Ile) are hydrophobic and therefore candidates for replacement. Of the 16 interface residues in the VH domain, H111(Leu), H114(pro), H41(pro), H84(val), H87(met) and H89(Ile) were identified as possible candidates for substitution by hydrophilic residues in the scFv fragment of the antibody 4-4-20 (Fig. 1 and Table 1).

Not all of these hydrophobic residues are equally good candidates for replacements, however. While residues which are hydrophobic in one particular sequence but hydrophilic in many other sequences may appear most attractive, the conserved hydrophobic residues listed in Table 1 have also been investigated, since the evolutionary pressure which kept these conserved residues acted on the Fab fragment within the whole antibody, but not the isolated Fv portion. In this study, we did not replace the proline residues since pro L40 and pro H41 form the hairpin turns at the bottom of the framework II region, while the conserved VL cis-proline L8 and proline residues H9 and H14 determine the shape of framework I of the immunoglobulin variable domains.

Excluding prolines, this leaves residues L9 (Leu in 4-4-20, ser in most kappa chains), L15 (Leu, usually hydrophobic), L83 (Leu, usually val or phe) and L106 (Ile, as in 86% of all kappa chains) in the VL domain and H111 (Leu as in 60% of all heavy chains), H84 (val, in other VH domains frequently ala or ser), H87 (met, usually ser) and H89 (Ile, most frequently val) in VH as possible candidates for replacement in the 4-4-20 scFv fragment.

#### (b) Mutations in the 4-4-20 scFv

For the 4-4-20 scFv fragment some of the crucial residues identified in the sequence analysis described above are already hydrophilic, but nevertheless 9 residues are of hydrophobic nature (including pro12 in the light chain) (Table 1). We chose three residues for closer analysis by mutations.

Leu15 in VL is a hydrophobic amino acid in 98 % of all kappa chains (Table 1). Leu11 is conserved in VH (Table 1) and is involved in v/c interdomain contacts (Lesk & Chothia, 1988). In contrast, valine occurs very infrequently at position H84: mainly found at this

-15-

position are threonine or serine and alanine (Table 1). As can be seen in Figure 1, val84 is contributing to a large hydrophobic patch at the newly exposed surface of V<sub>H</sub>. All three positions were mutated into acidic residues, and L11 was also changed to asparagine (Table 2).

The scFv fragment was tested and expressed with two different linkers, the 15-mer linker (Gly4Ser)<sub>3</sub> (Huston et al., 1995) and the same motif extended to 30 amino acids (Gly4Ser)<sub>6</sub>. All mutations were tested in both constructs. The *in vivo* results of the different mutations on solubility were identical, and therefore only the results of the 30-mer linker are described in more detail. The periplasmic expression experiments were carried out at 24.5°C, and all constructs were tested for soluble and insoluble protein by immunoblotting. The ratio of insoluble to soluble (i/s) protein was determined for every mutant. In Figure 3 A-D, insoluble (lane 1) and soluble (lane 2) fractions of the wt scFv are shown. Nearly no soluble material occurs in periplasmic expression, which is consistent with previous reports of Bedzyk et al. (1990) and Denzin et al. (1991), who described earlier that the periplasmic expression of the wt scFv leads mainly to periplasmic inclusion bodies.

The single point mutation L15E in V<sub>L</sub> (Flu1) shows no effect on the ratio i/s when compared with the wt (Fig. 3A, lane 3, 4). Mutating leu at position 11 in the heavy chain to asparagine (Flu2) also shows nearly no effect compared to the wt, whereas the substitution with aspartic acid (Flu3) changes the i/s ratio to more soluble protein, but still this effect is not very dramatic. In contrast, the point mutation at position 84 (Flu4, Fig. 3B, lane 3, 4 and Fig 3D, lane 3, 4) had a dramatic influence on the solubility of the scFv fragment of the antibody 4-4-20. The ratio i/s is changed to about 1:1, resulting in a 25-fold increase of soluble protein compared to the wt.

The combination of V84D with L11N or L11D (Flu5, Flu6) also changes the ratio i/s compared to the wt, but this ratio compared to V84D alone is not improved further (Fig. 3B). Interestingly, the combination of Flu5 with the light chain mutation at position 15 (Flu9) leads to less soluble material (Fig. 3C lane 7,8) than Flu5 itself (Fig. 3B, lane 5, 6). The negative influence of the L15E mutations can also be seen in Flu8 (Fig. 3C, lane 5, 6) compared with

Flu3 (Fig. 3A, lane 7, 8). In Fig. 3D the comparison of the wt (lane 1, 2 and 5, 6) and Flu4 (lane 3, 4 and 7, 8) is shown in both the 15-mer and the 30-mer construct.

The negative effect of L15E can be rationalized by looking at a model of the 4-4-20 scFv fragment. L15 is forming a hydrophobic pocket together with residues A80, L83, and L106. Apparently, L15 stabilizes the scFv fragment by hydrophobic interactions with its closest neighbours. Thus the exchange L15E for making the scFv fragment more hydrophilic and more soluble is made at the expense of the fragment stability. The analysis of hydrophobic interactions within a fragment should thereby be used to choose the solvent-exposed residues to be mutated in the case of any other antibody fragment.

Combinations of various serine mutations in VH led to further improvements in the i/s ratio. The mutants FH15 (V84S, M87S, I89S) and FH20 (L11S, V84S, M87S, I89S) both showed more than 70% of soluble protein in immunoblots (data not shown).

The negative effect of L15E

#### (c) Functional expression and purification

The oligomerization of scFv fragments as a function of linker length has been investigated previously. A continuous decrease in the amount of dimer and multimer formation as a function of linker length has been reported (Desplancq et al., 1994; Whitlow et al., 1994). While the (Gly4Ser)<sub>3</sub> linker has been shown to lead to monomeric scFvs in many cases in the VH-VL direction, this is often not the case in the VL-VH direction. This is caused by an asymmetry in the VL/VH arrangement, leading to a longer distance between the end of VH and the N-terminus of VL than between C-terminus of VL and N-terminus of VH (Huston et al., 1995). Consequently, a linker of identical length may lead to different properties of the resulting molecules.

Since we have chosen to use the minimal perturbation FLAG (Knappik & Plückthun, 1994) at the N-terminus of VL in our constructs and thus the VL-linker-VH orientation, we have investigated the use of longer linkers. In the periplasmic expression in *E. coli* no difference between the 15-mer and the 30-mer linker in the corresponding mutants is visible (Fig. 3D), but when we attempted to purify the two Flu4 scFvs with long and short linker, a big

discrepancy between the two constructs was found. The purification of the Flu4 mutant (V84D) with the 15-mer linker leads to very small amounts of partially purified protein (about 0.015 mg per liter and OD; estimated from SDS-PAGE after IMAC purification), whereas the 30-mer linker construct gives about 0.3 mg per liter and OD of highly pure functional protein. All mutants with 30-mer linker were tested in gel filtration and found to be monomeric (data not shown).

For further *in vitro* characterization five mutants were purified with the 30-mer linker, V84D (Flu4), V84D/L11D (Flu6), L11D (Flu3), and the serine mutants FH15 and FH20 (see iii(b)). A two-step chromatography, first using IMAC and then cation-exchange chromatography, led to homogeneous protein. The *i/s* ratio of the antibody fragments (Fig. 3) was also reflected in the purification yield of functional protein. The highly soluble mutant Flu4 (V84D) (Fig. 3B lane 3, 4) yielded about 0.3 mg purified and functional protein per liter and OD, Flu6 (L11D/V84D) (Fig. 3B lane 7, 8) yielded about 0.25 mg per liter and OD and Flu3 (less soluble material on the blot in Fig. 3A lane 7, 8) yielded 0.05 mg per liter and OD. The serine mutants FH15 and FH20 yielded 0.3 mg and 0.4 mg per liter and OD, respectively. The wt scFv of the antibody 4-4-20 did not give any soluble protein at all in periplasmic expression with either linker, and it was therefore expressed as cytoplasmic inclusion bodies, followed by refolding *in vitro* and fluorescein affinity chromatography. The refolded wt scFv was shown by gel filtration to be monomeric with the 30-mer linker (data not shown).

#### (d) Biophysical properties of the mutant scFvs

Since we changed amino acids which are conserved, it cannot be excluded that changes at these positions may be transmitted through the structure and have an effect on the binding constant, even though they are very far from the binding site (Chatellier et al., 1996). To eliminate this possibility, we determined the binding constant of the mutants Flu3, Flu4, Flu6 and the wt scFv. Fluorescence titration was used to determine  $K_D$  in solution by using the quenching of the intrinsic fluorescence of fluorescein when it binds to the antibody. The fluorescence quenching at 510 nm was measured as a function of added scFv. The  $K_D$  values (Table 3 and Fig. 4) obtained for all three mutant scFvs and the wt scFv are very similar and

correspond very well to the recently corrected  $K_D$  of the monoclonal antibody 4-4-20 (Miklasz et al., 1995).

To determine whether the mutations had an influence on the thermodynamic stability of the protein we determined the equilibrium unfolding curves by urea denaturation. V84D mutant and the wt scFv were used for this analysis, and in Figure 5 an overlay plot is shown. The midpoint of both curves is at 4.1 M urea. Both curves were fitted by an algorithm for a two-state model described by Pace (1990), but the apparent small difference between the V84D mutant and the wt scFv is not of statistical significance.

Aggregation of folding intermediates could be another explanation for the different *in vivo* results between the mutant scFvs and the wt scFv (Fig.3). In the periplasm of *E. coli*, the protein concentrations are assumed to be rather high (van Wielink & Duine, 1990) and the aggregation effects could thus be pronounced. In order to estimate the aggregation behavior *in vitro*, we have measured the thermal aggregation rates at different temperatures. In Figure 6 it is clearly seen that the wt scFv is significantly aggregating already at 44°C, whereas the mutant V84D tends to aggregate more slowly. The wt scFv is thus clearly more aggregation prone than the mutant scFv. This is very similar to the observations made with different mutations on the antibody McPC603 (Knappik and Plückthun, 1995), where no correlation was found between equilibrium denaturation curves and expression behavior, but a good correlation was found with the thermal aggregation rates.

## Figures and Tables

Figure 1: Space-filling representation of the Fv fragment of the antibody  
4-4-20

Figure 2: Variable/constant domain interface residues for  $V_L$  (2a) and  $V_H$  (2b). For 30 non-redundant Fab fragments taken from the Brookhaven Databank, the solvent accessible surface of the amino acid side chains was calculated in the context of

an Fv and of an Fab fragment. The plot shows the relative reduction in accessible surface upon contact with the constant domains (overlay plot for all 30 Fv fragments). In the sequence alignment, residues contributing to the v/c interface are highlighted. The symbols indicate the relative reduction of solvent accessible surface upon removing the constant domains (symbols: no symbol < 1%: ● < 20%: ■ < 40%: ▲ < 60%: ▼ < 80%, and ◆ ≥ 80%). Circles indicate those positions which are further analyzed (see Table 1).

**Figure 3:** Western blots showing the insoluble (i) and soluble (s) fractions of cell extracts, prepared as described in Material and Methods, expressing the scFv fragments of the antibody 4-4-20. The amino acids substituted in the various mutants are given in Table 2.

**Figure 4:** Scatchard plot of the fluorescence titration of fluorescein (20 nM) with antibody (4 to 800 nM), measured at 510 nm. The value  $r$  was obtained from  $(F-F_0)/(F_\infty-F_0)$ , where  $F$  is the measured fluorescein fluorescence at a given antibody concentration,  $F_0$  is the fluorescence in the absence of antibody and  $F_\infty$  when antibody is present in large excess. Note that  $r$  gives the saturation of fluorescein by antibody. (a) Titration of wt scFv. (b) titration of Flu4 (V84D).

**Figure 5:** An overlay plot of the urea denaturation curves is shown. (X) wt scFv. (o) Flu4.

**Figure 6:** Thermal denaturation time courses at 40 and 44°C for wt and Flu4 scFv fragment are shown. (a) wt scFv at 40°C. (b) Flu4 at 40°C. (c) Flu4 at 44°C. (d) wt scFv at 44°C.

**Table 1:** Sequence variability of residues contributing to the v/c interface: Residue statistics are based on the variable domain sequences in the Kabat database (March 1996). Sequences which were <90% complete were excluded from the analysis. Number of sequences analyzed: human VL kappa: 404 of 881, murine VL kappa: 1061 of 2239, human VL lambda: 223 of 409, murine VL lambda: 71 of 206, human VH: 663 of 1756, murine VH: 1294 of 3849. Position refers to the sequence position

-20-

according to Kabat et al. 1991. %exp. (Fab) to the relative side chain accessibility in an Fab fragment as calculated by the program NACCESS (NACCESS v2.0 by Simon Hubbard (<http://www.biochem.ucl.ac.uk/~roman/naccess/naccess.html>)). %exp. (ind.) to the relative side chain accessibility in the isolated VL or VH domain. %buried to the relative difference in side chain accessibility between Fv and Fab fragment. Consensus refers to the sequence consensus, and Distribution to the distribution of residue types.

Table 2: Mutations introduced in the scFv fragment of the antibody 4-4-20: Each line represents a different protein carrying the mutations indicated. The residues are numbered according to Kabat et al. (1991).

Table 3:  $K_D$  values of the different scFv mutants determined in fluorescence titration: The  $K_D$  values are given in nM, the error was calculated from the Scatchard analysis (Fig. 4). # determined by Miklasz et al. (1995)

### References

Better, M., Chang, P., Robinson, R. & Horwitz, A.H. (1988). *E. coli* secretion of an active chimeric antibody fragment. *Science* **240**, 1041-1043.

Bird, R.E., Hardman, K.D., Jacobson, J.W., Johnson, S., Kaufman, B.M., Lee S.M., Lee T., Pope S.H., Riordan G.S., & Whitlow M. (1988). Single-chain antigen-binding proteins [published erratum appears in (1989). *Science* **244**, 409]. *Science* **242**, 423-6.

Bedzyk, W.D., Weidner, K.M., Denzin, L.K., Johnson, L.S., Hardman, K.D., Pantoliano, M.W., Asel, E.D. & Voss, E.W., Jr. (1990). Immunological and structural characterization of a high affinity anti-fluorescein single-chain antibody. *J. Biol. Chem.* **265**, 18615-18620.

Blake, M.S., Johnston, K.H., Russel-Jones, G.J. & Gotschlich, E.C. (1984). A rapid, sensitive method for detection of alkaline phosphatase-conjugated anti-antibody on Western blots. *Anal. Biochem.* **136**, 175-179.

Brinkmann, U., Reiter, Y., Jung, S., Lee, B. & Pastan, I. (1993). A recombinant immunotoxin containing a disulfide-stabilized Fv fragment. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 7538-7542.

Carter, P., Kelley, R.E., Rodrigues, M.L., Snedecor, B., Covarrubias, M., Velligan, M.D., Wong, W.L.T., Rowland, A.M., Kotts, C.E., Carver, M.E., Yang, M., Bourell, J.H., Shepard, H.M. & Henner, D. (1992). High level *Escherichia coli* expression and production of a bivalent humanized antibody fragment. *Bio/Technology* **10**, 163-167.

Casari, G. & Sippl, M.J. (1992). Structure-derived hydrophobic potential. Hydrophobic potential derived from X-ray structures of globular proteins is able to identify native folds. *J. Mol. Biol.* **224**, 725-32.

Chatellier, J., van Regenmortel, M.H.V., Vernet, T. & Altschuh, D. (1996). Functional mapping of conserved residues located at the VL and VH domain interface of an Fab. *J. Mol. Biol.*, in press.

Denzin, L.K., Whitlow, M. & Voss, E.W., Jr. (1991). Single-chain site-specific mutation of fluorescein-amino acid contact residues in high affinity monoclonal antibody 4-4-20. *J. Biol. Chem.* **266**, 14095-14102.

Desplancq, D., King, D.J., Lawson, A.D. & Mountain, A. (1994). Multimerization behaviour of single chain Fv variants for the tumour-binding antibody B72.3. *Protein Eng.* **7**, 1027-1033.

Ge, L., Knappik, A., Pack, P., Freund, C. & Plückthun, A. (1995). *Expressing antibodies in Escherichia coli*. Antibody Engineering. A Practical Approach (Ed. C.A.K. Borrebaeck). IRL Press. Oxford. pp. 229-266.

Gething, M.J. & Sambrook, J. (1992). Protein folding in the cell. *Nature* **355**, 33-45.

Gill, S.C. & von Hippel, P.H. (1989). Calculation of protein extinction coefficients from amino acid sequence data. *Anal. Biochem.* **182**, 319-326.

Glockshuber, R., Malia, M., Plitzinger, I. & Plückthun, A. (1992). A comparison of strategies to stabilize immunoglobulin Fv-fragments. *Biochemistry* **29**, 1362-1366.

Hiatt, A. (1990). Antibodies produced in plants. *Nature* **344**, 469-470.

Hiatt, A. & Ma, J. K. (1993). Characterization and applications of antibodies produced in plants. *Int. Rev. Immunol.* **10**, 139-152.

Ho, S.N., Hunt, H.D., Horton, R.M., Pullen, J.K. & Pease, L.R. (1989). Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* **77**, 51-9.

Hochuli, E., Bannwarth, W., Döbeli, H., Gentz, R. & Stüber, D. (1988). Genetic approach to facilitate purification of recombinant proteins with a novel metal chelate adsorbent. *Bio/Technology* **6**, 1321-1325.

Hopp, T.P., Prickett, K.S., Price, V.L., Libby, R.T., March, C.J., Cerretti, D.P., Urdal, D.L. & Conlon, P.J. (1988). A short polypeptide marker sequence useful for recombinant protein identification and purification. *Bio/Technology* **6**, 1204-1210.

Horwitz, A. H., Chang, C. P., Better, M., Hellstrom, K. E. & Robinson, R. R. (1988). Secretion of functional antibody and Fab fragment from yeast cells. *Proc. Natl. Acad. Sci. U.S.A.* **85**, 8678-8682.

Huston, J., George, A.J.T., Tai, M., McCartney, J.E., Jin, D., Segal, D.M., Keck, P. & Oppermann, H. (1995). *Single-chain Fv design and production by preparative folding*. Antibody Engineering. A Practical Approach (Ed. C.A.K. Borrebaeck). IRL Press, Oxford, pp. 185-228.

Huston, J.S., Levinson, D., Mudgett-Hunter, M., Tai, M.S., Novotny, J., Margolies, M.N., Ridge, R.J., Brucolieri, R.E., Haber, E. & Crea, R. (1988). Protein engineering of antibody binding sites. recovery of specific activity in an anti-digoxin single-chain Fv analogue produced in *Escherichia coli*. *Proc. Natl. Acad. Sci. U. S. A.* **85**, 5879-83.

Johnson, K.S., Jackson, R.H. & Chiswell, D.J. (1992). Binding Domains. *PCT Application WO 92/01787*.

Kabat, E.A., Wu, T.T., Perry, H.M., Gottesmann, K.S. & Foeller, C. (1991). Sequences of proteins of immunological interest. U.S. Dept. of Health and Human Services. Public Health Service. National Institutes of Health. NIH Publication 91-3242.

Knappik, A. & Plückthun, A. (1994). An improved affinity tag based on the FLAG peptide for detection and purification of recombinant antibody fragments. *BioTechniques* **17**, 754-761.

-23-

Knappik, A. & Plückthun, A. (1995). Engineered turns of a recombinant antibody improve its *in vivo* folding. *Protein Eng.* **8**, 81-89.

Kunkel, T.A., Bebenek, K. & McClary, J. (1991). Efficient site-directed mutagenesis using uracil-containing DNA. *Methods in Enzymol.* **204**, 125-39.

Kunkel, T.A., Roberts, J.D. & Zakour, R.A. (1987). Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods in Enzymol.* **154**: 367-382.

Lee, B. & Richards, F.M. (1971). The interpretation of protein structures: estimation of static accessibility. *J. Mol. Biol.* **55**, 379-400.

Lesk, A.M. & Chothia, C. (1988). Elbow motion in the immunoglobulins involves a molecular ball-and-socket joint. *Nature (London)* **335**, 188-190.

Leistler, B. & Perham, R.N. (1994). Solubilizing buried domain proteins: A self-assembling interface domain from glutathione reductase. *Biochemistry* **33**, 2773-2781.

Lindner, P., Guth, B., Wülfing, C., Krebber, C., Steipe, B., Müller, F. & Plückthun, A. (1992). Purification of native proteins from the cytoplasm and periplasm of *Escherichia coli* using IMAC and histidine tails: a comparison of proteins and protocols. *Methods: A Companion to Methods Enzymol.* **4**, 41-56.

Miklasz, S.D., Gulliver, G.A. & Voss, E.W., Jr. (1995). High-affinity rat anti-fluorescein monoclonal antibody with unique fine specificity properties including differential recognition of dynamic ligand analogues. *J. Mol. Recognition* **8**, 258-269.

Munro, S. & Pelham, H.R.B. (1986). An Hsp70-like protein in the ER: identity with the 78 kd glucose-regulated protein and immunoglobulin heavy chain binding protein. *Cell*, **46**, 291-300.

Nozaki, Y. & Tanford, C. (1971). The solubility of amino acids and two glycine peptides in aqueous ethanol and dioxane solutions. Establishment of a hydrophobicity scale. *J. Biol. Chem.* **246**, 2211-7.

Nyyssönen, E., Penttila, M., Harkki, A., Saloheimo, A., Knowles, J. K. & Keraanen, S. (1993). Efficient production of antibody fragments by the filamentous fungus *Trichoderma reesei*. *Bio/Technology* **11**, 591-595.

Pace, C.N. (1990). Measuring and increasing protein stability. *Trends Biotechnol.* **8**, 93-98.

Pack, P. & Plückthun, A. (1992). Miniantibodies: use of amphipathic helices to produce functional, flexibly linked dimeric Fv fragments with high avidity in *Escherichia coli*. *Biochemistry* **31**, 1579-1584.

Pack, P., Kujau, M., Schroeckh, V., Knüpfer, U., Wenderoth, R., Riesenber, D. & Plückthun, A. (1993). Improved bivalent miniantibodies, with identical avidity as whole antibodies, produced by high cell density fermentation of in *Escherichia coli*. *Bio/Technology* 11, 1271-1277.

Pack, P. (1994). Mini-Antikörper: Bivalente, tetravalente und bispezifische Immunglobuline aus *E. coli*. Ph.D. thesis, Ludwig-Maximilians-Universität München.

Plückthun, A. (1992). Mono- and bivalent antibody fragments produced in *Escherichia coli*: engineering, folding and antigen binding. *Immun. Rev.* 130, 151-188.

Plückthun, A., Krebber, A., Krebber, C., Horn, U., Knüpfer, U., Wenderoth, R., Nieba, L., Proba, K. & Riesenber, D. (1996). *Producing antibodies in Escherichia coli: From PCR to fermentation*. A practical approach. Antibody Engineering (Ed. J. McCafferty). IRL Press, Oxford, pp. 203-252.

Potter, K. N., Li, Y. & Capra, J. D. (1993). Antibody production in the baculovirus expression system. *Int. Rev. Immunol.* 10, 103-112.

Prickett, K.S., Amberg, D.C. & Hopp, T.P. (1989). A calcium-dependent antibody for identification and purification of recombinant proteins. *BioTechniques* 7, 580-589.

Prodromou, C. & Pearl, L.H. (1992). Recursive PCR: a novel technique for total gene synthesis. *Protein Eng.* 5, 827-829.

Ridder, R., Schmitz, R., Legay, F. & Gram, H. (1995). Generation of rabbit monoclonal antibody fragments from a combinatorial phage display library and their production in the yeast *Pichia pastoris*. *Bio/Technology* 13, 255-260.

Rose, G.D. & Wolfenden, R. (1993). Hydrogen bonding, hydrophobicity, packing, and protein folding. *Annu Rev. Biophys. Biomol. Struct.* 22, 381-415.

Rosenberg, S.A. & Lotze, M.T. (1986). Cancer immunotherapy using interleukin-2 and interleukin-2 activated lymphocytes. *Ann. Rev. Immunol.* 4, 681-709.

Skerra, A. & Plückthun (1988). Assembly of a functional immunoglobulin Fv fragment in *Escherichia coli*. *Science* 240, 1038-1041.

Studier, F.W. & Moffatt, B.A. (1986). Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J. Mol. Biol.* 189, 113-130.

Trill, J. J., Shatzman, A. R. & Ganguly, S. (1995). Production of monoclonal antibodies in COS and C110 cells. *Curr. Opin. Biotechnol.* 6, 553-560.

-25-

Trower, M.K. (1994). Site-directed mutagenesis using a uracil-containing phagemid template. *Methods Mol. Biol.* **31**, 67-77.

Ullrich, H.D., Patten, P.A., Yang, P.L., Romesberg, F.E. & Schultz, P.G. (1995). Expression studies of catalytic antibodies. *Proc. Natl. Acad. Sci. USA* **92**, 11907-11911.

Van Wielink, J.E. & Duine, J.A. (1990). How big is the periplasmic space. *Trends Biochem. Sci.* **15**, 136-137.

Vitetta, E.S., Thorpe, P.E. & Uhr, J. (1993). Immunotoxins: magic bullets or misguided missiles. *Immunol. Today* **14**, 253-259.

Viville, S. (1994). Site-directed mutagenesis using a double-stranded DNA template. *Methods Mol. Biol.* **31**, 57-65.

Ward, V. K., Kreissig, S. B., Hammock, B. D. & Choudary, P. V. (1995). Generation of an expression library in the baculovirus expression vector system. *J. Virol. Methods* **53**, 263-272.

Whitelam, G. C., Cockburn, W. & Owen, M. R. (1994). Antibody production in transgenic plants. *Biochem. Soc. Trans.* **22**, 940-944.

Whitlow, M., Filupa, D., Rollence, M.L., Feng, S. & Wood, J.F. (1994). Multivalent Fvs: characterization of single-chain Fv oligomers and preparation of a bispecific Fv. *Protein Eng.* **7**, 1017-1026.

Wu, X. C., Ng, S. C., Near, R. I. & Wong, S. L. (1993). Efficient production of a functional single-chain antidigoxin antibody via an engineered *Bacillus subtilis* expression-secretion system. *Bio/Technology* **11**, 71-76.

**Claims**

1. A DNA sequence which encodes an immunoglobulin superfamily (IgSF) domain or fragment which differs from a parent IgSF domain or fragment in that the region which comprised or would comprise the interface with a second domain adjoined to said parent IgSF domain or fragment within the chain of a larger IgSF fragment or protein is made more hydrophilic by modification.
2. The DNA sequence according to claim 1 in which said modification is substitution of one or more amino acids at said interface with amino acids which are more hydrophilic.
3. The DNA sequence according to claim 1 in which said modification is insertion of one or more hydrophilic amino acids in said interface, or insertion of amino acids which increase the overall hydrophilicity in said interface, or deletion of one or more hydrophobic amino acids in said interface, or deletion of amino acids, said deletion leading to an increase in the overall hydrophilicity in said interface.
4. The DNA sequence according to claim 1 in which said modification consists of any two or more of:
  - a) substitution of one or more amino acids at said interface with amino acids which are more hydrophilic.
  - b) insertion of one or more hydrophilic amino acids in said interface, or insertion of amino acids which increase the overall hydrophilicity in said interface.
  - c) deletion of one or more hydrophobic amino acids in said interface, or deletion of amino acids, said deletion leading to an increase in the overall hydrophilicity in said interface.
5. The DNA sequence according to any of claims 2 to 4 in which said substituted or inserted amino acid is taken from the list Asn, Asp, Arg, Gln, Glu, Gly, His, Lys, Ser, and Thr.
6. The DNA sequence according to any of claims 1 to 5 in which said parent IgSF domain is part of an IgSF fragment.

-27-

7. The DNA sequence according to any of claims 1 to 6 in which said domain or fragment is derived from an antibody.
8. The DNA sequence according to claim 7 in which said fragment is a Fab fragment.
9. The DNA sequence according to claim 7 in which said fragment is an Fv fragment.
10. The DNA sequence according to claim 7 in which said fragment is a scFv fragment.
11. The DNA sequence according to claim 7 in which said fragment is an Fv stabilized by an inter-domain disulphide bond.
12. The DNA sequence according to any of claims 9 to 11 in which said interface region comprises residues 9, 10, 12, 15, 39, 40, 41, 80, 81, 83, 103, 105, 106, 106A, 107, 108 for VL, and residues 9, 10, 11, 13, 14, 41, 42, 43, 84, 87, 89, 105, 108, 110, 112, 113 for VH.
13. The DNA sequence according to any of claims 1 to 12, having a contiguous sequence which encodes one or more additional moieties.
14. The DNA sequence according to claim 13 in which at least one of said additional moieties is a toxin, a cytokine, or a reporter enzyme.
15. The DNA sequence according to claim 13 in which at least one of said additional moieties is at least part of a surface protein of an organism.
16. The DNA sequence according to claim 15 in which said organism is a filamentous bacteriophage.
17. The DNA sequence according to claim 16 in which said surface protein is the genell protein.
18. The DNA sequence according to claim 13 in which at least one of said additional moieties is capable of binding a metal ion.
19. The DNA sequence according to claim 18 in which at least one of said additional moieties comprises at least five histidines.
20. The DNA sequence according to claim 13 in which said moiety is a peptide.

-28-

21. The DNA sequence according to claim 20 in which said peptide is a labelling tag.
22. The DNA sequence according to claim 21 in which said labelling tag is c-myc or FLAG.
23. The DNA sequence according to claim 20 in which said peptide comprises an association domain which results in self-association of two or more of said antibody fragments.
24. The DNA sequence according to claim 23 in which said association domain is derived from a leucine zipper or from a helix-turn-helix motif.
25. The DNA sequence according to claim 20 in which said peptide comprises a first association domain which results in hetero-association of one or more of said antibody fragments with one or more peptides or proteins comprising a second hetero-association domain being able to associate with said first hetero-association domain.
26. A vector comprising a DNA sequence according to any of claims 1 to 25.
27. A host cell comprising a vector according to claim 26.
28. An IgSF domain or fragment, or a fusion protein comprising an IgSF domain or fragment, encoded by a DNA sequence according to any of claims 1 to 25, by a vector according to claim 26, or produced by a host cell according to claim 27.
29. A diagnostic composition comprising an IgSF domain or fragment, or a fusion protein comprising an IgSF domain or fragment, according to claim 28.
30. A therapeutic composition comprising an IgSF domain or fragment, or a fusion protein comprising an IgSF domain or fragment, according to claim 28.
31. A method for deriving a DNA sequence according to any of claims 1 to 25 which comprises the following steps:
  - i) analyzing the interface region of a parent IgSF domain for hydrophobic residues which are solvent-exposed.
  - ii) identifying one or more of the hydrophobic residues to be substituted by more hydrophilic residues, or one or more positions where hydrophilic residues or amino acid stretches enhancing the overall hydrophilicity of the interface region can be

inserted into said interface region, or one or more positions where hydrophobic residues or amino acid stretches enhancing the overall hydrophobicity of the interface region can be deleted from said interface region, or any combination of said substitutions, said insertions, and said deletions to give one or more mutants of said parent IgSF domain.

32. A method for making an IgSF domain or fragment, or a fusion protein comprising an IgSF domain or fragment, according to claim 28 which comprises the following steps:

- i) deriving a DNA sequence according to claim 31.
- ii) preparing DNA encoding said mutant or mutants, said DNA being prepared either separately or as a mixture.
- iii) introducing said DNA or DNA mixture in a vector system suitable for expression of said mutant or mutants, said vector system optionally comprising one or more additional DNA sequences suitable for expression of additional IgSF domains or fragments, or one or more DNA sequences suitable for expression of a fusion protein comprising said mutant or mutants, or any combination of said additional DNA sequences.
- iv) introducing said vector system into suitable host cells and expressing said mutant or mixture of mutants, or expressing said mutants or mixture of mutants in combination with the expression products of said additional DNA sequences.
- v) identifying and characterizing one or more mutants, alone or in said combination, which are obtained in higher yield in soluble form, and
- vi) if necessary, repeating steps ii) to vi) to increase the hydrophilicity of said identified mutant or mutants, alone or in said combination, further.

33. The method according to claim 32 in which said host is a bacterium, a fungus, a plant, an insect cell, or a cell-line of mammalian origin.
34. A method for the production of an IgSF domain or fragment of claim 28 comprising culturing a host cell of claim 27 and isolating said domain or fragment.

**Figure 1: Space filling representation of the Fv fragment of the antibody 4-4-20**

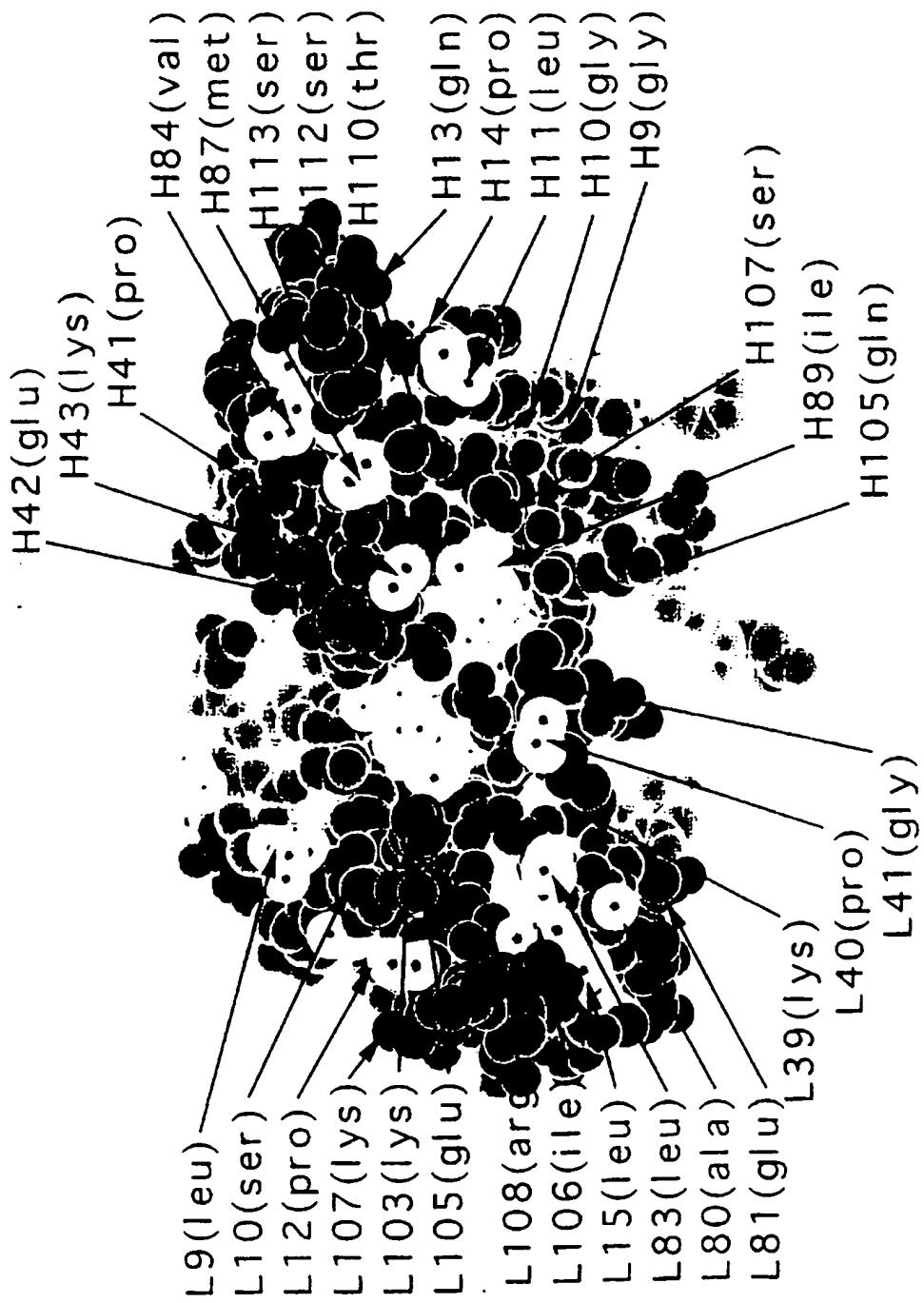
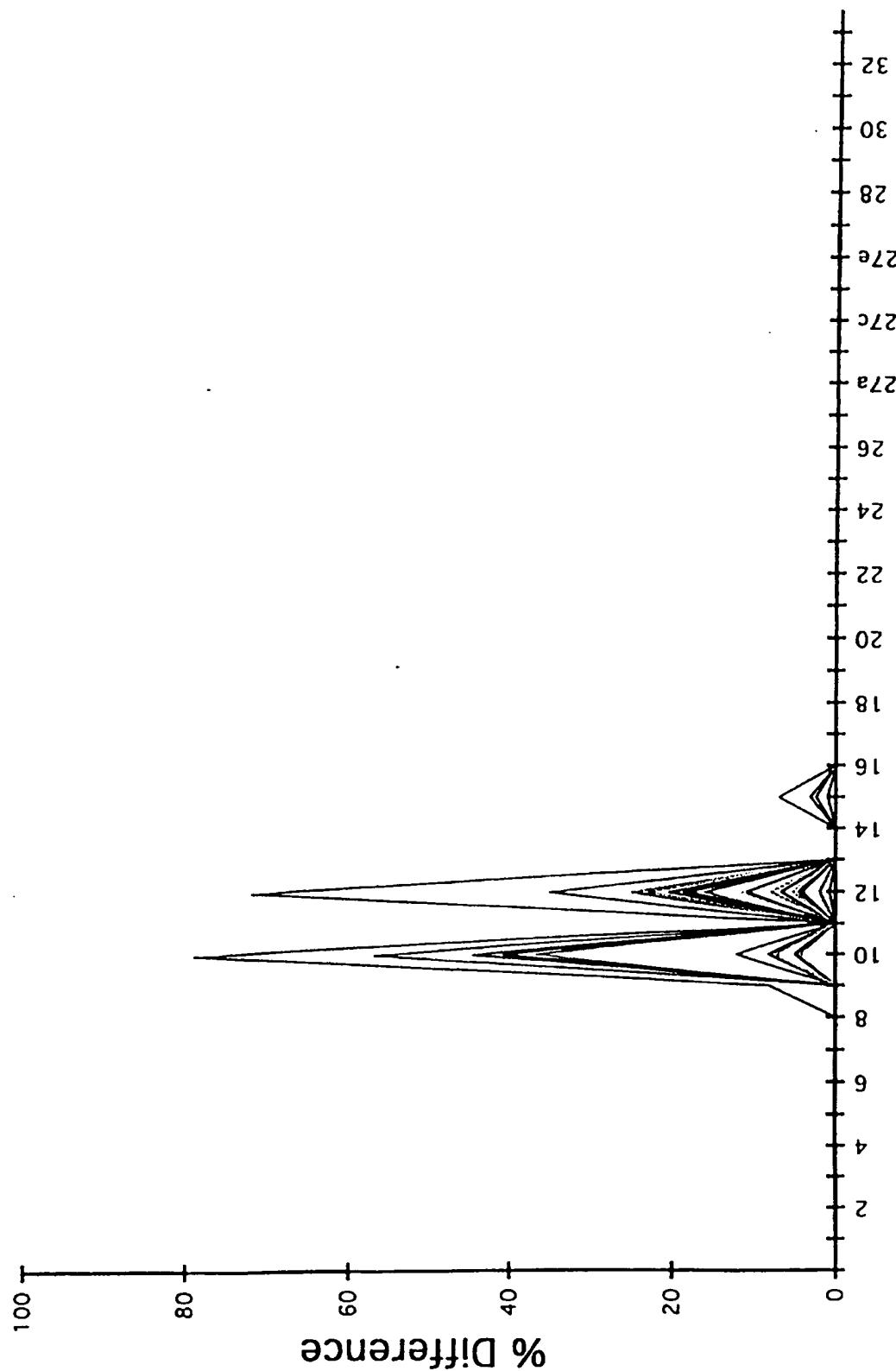
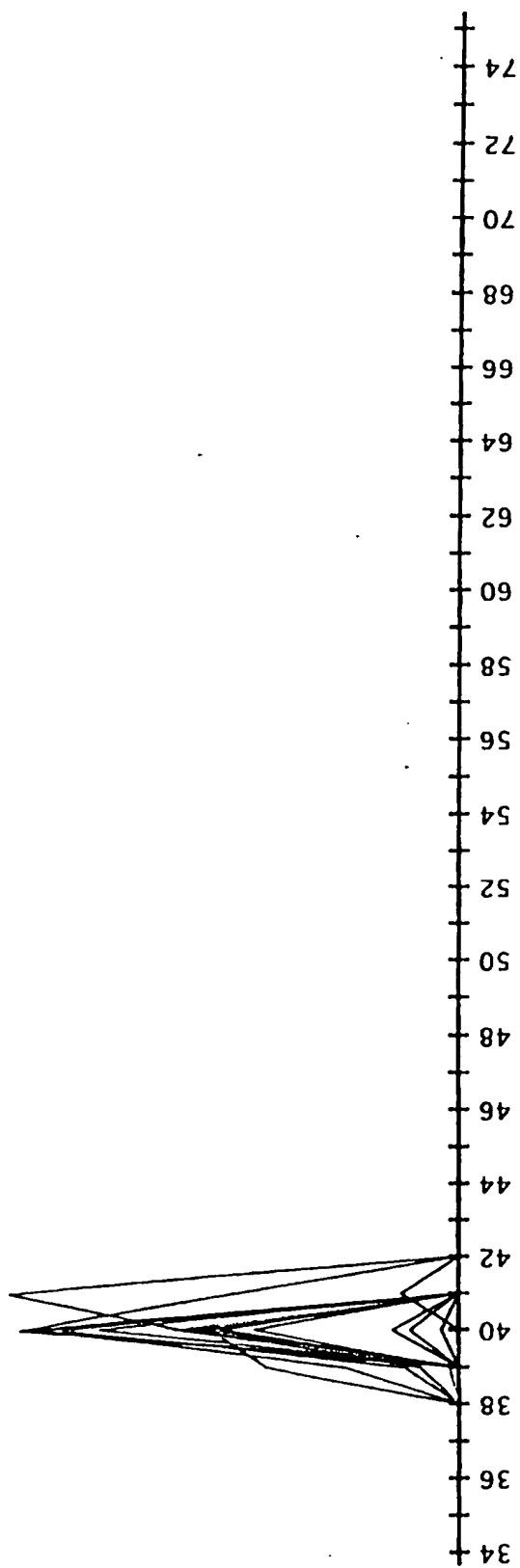


Figure 2a: Variable/constant domain interface residues for VL



**Figure 2a: Variable/constant domain interface residues for VL  
(cont.)**



**Figure 2a: Variable/constant domain interface residues for VL (cont.)**

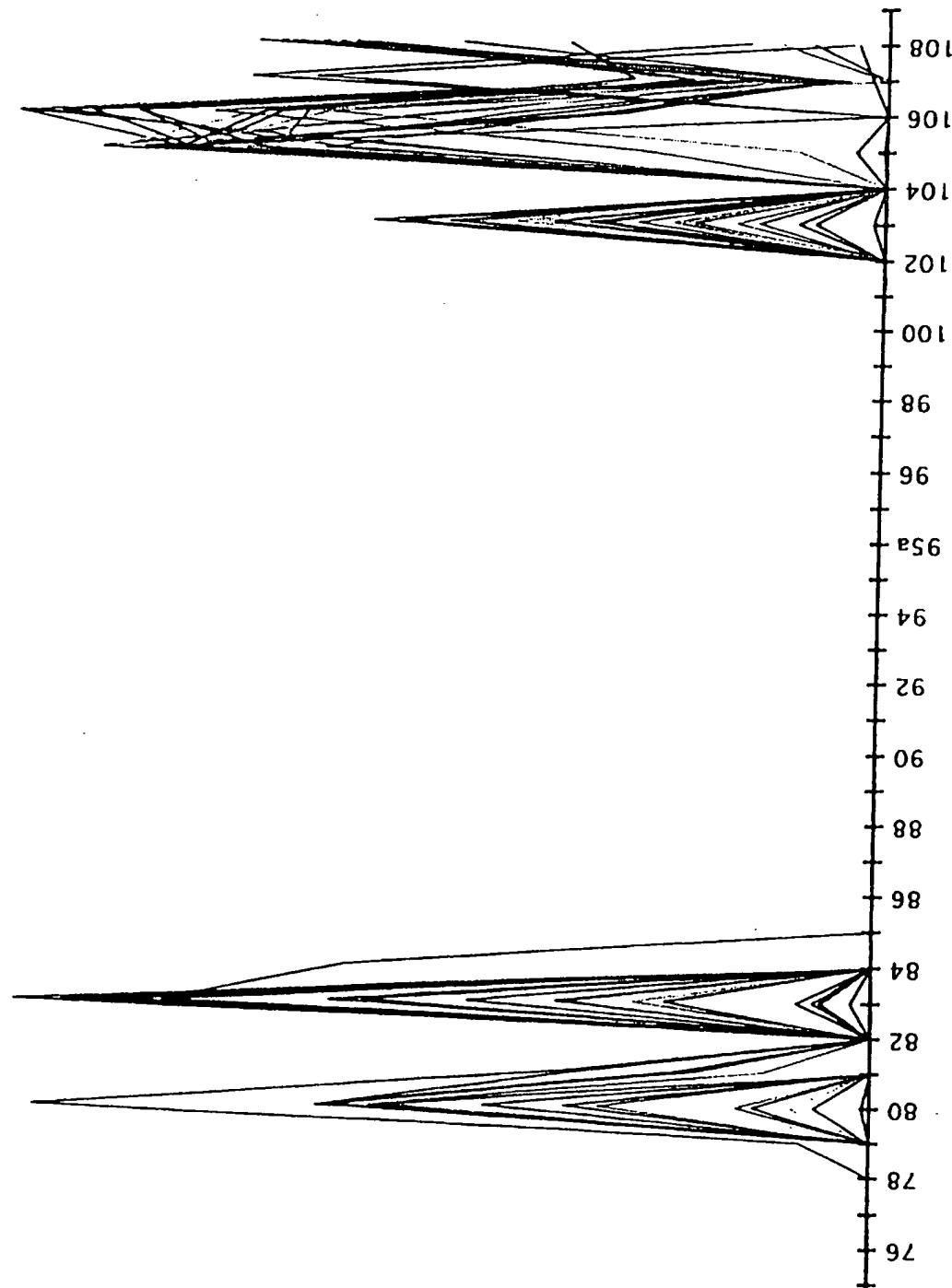
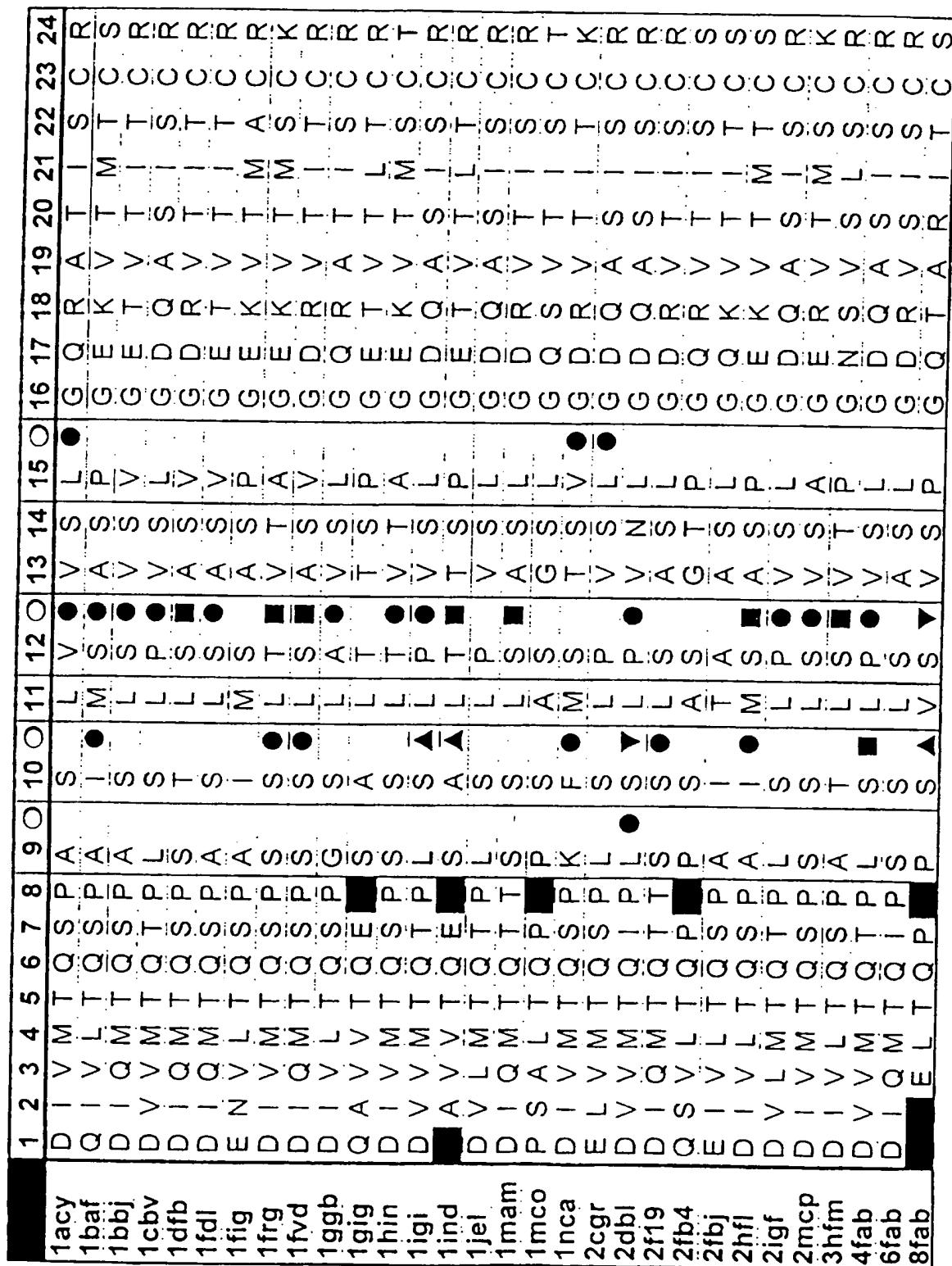
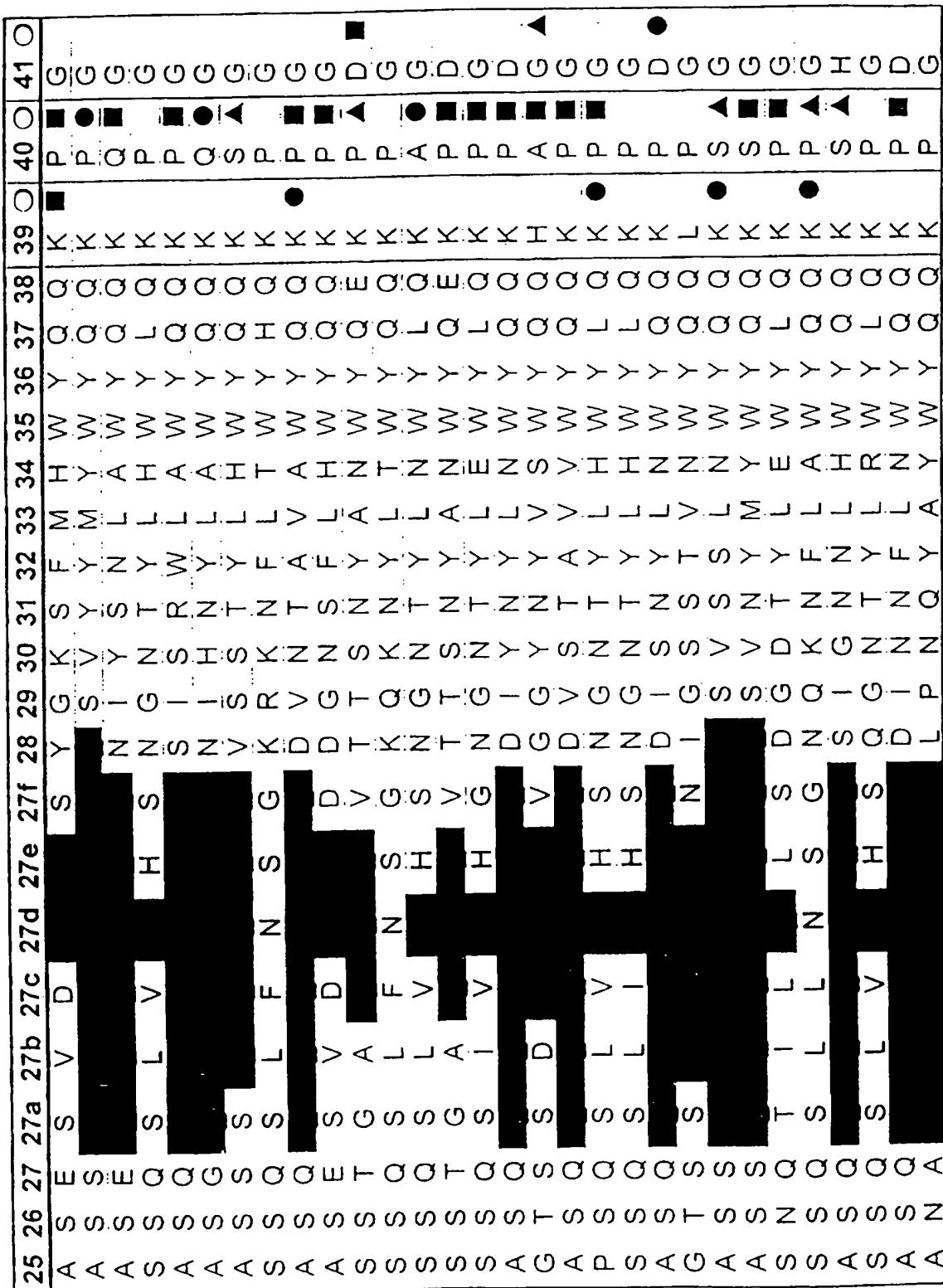


Figure 2a: Variable/constant domain interface residues for VL  
(cont.)



**Figure 2a: Variable/constant domain interface residues for VL (cont.)**



**Figure 2a: Variable/constant domain interface residues for VL (cont.)**

**Figure 2a: Variable/constant domain interface residues for VL (cont.)**

**Figure 2a: Variable/constant domain interface residues for VL (cont.)**

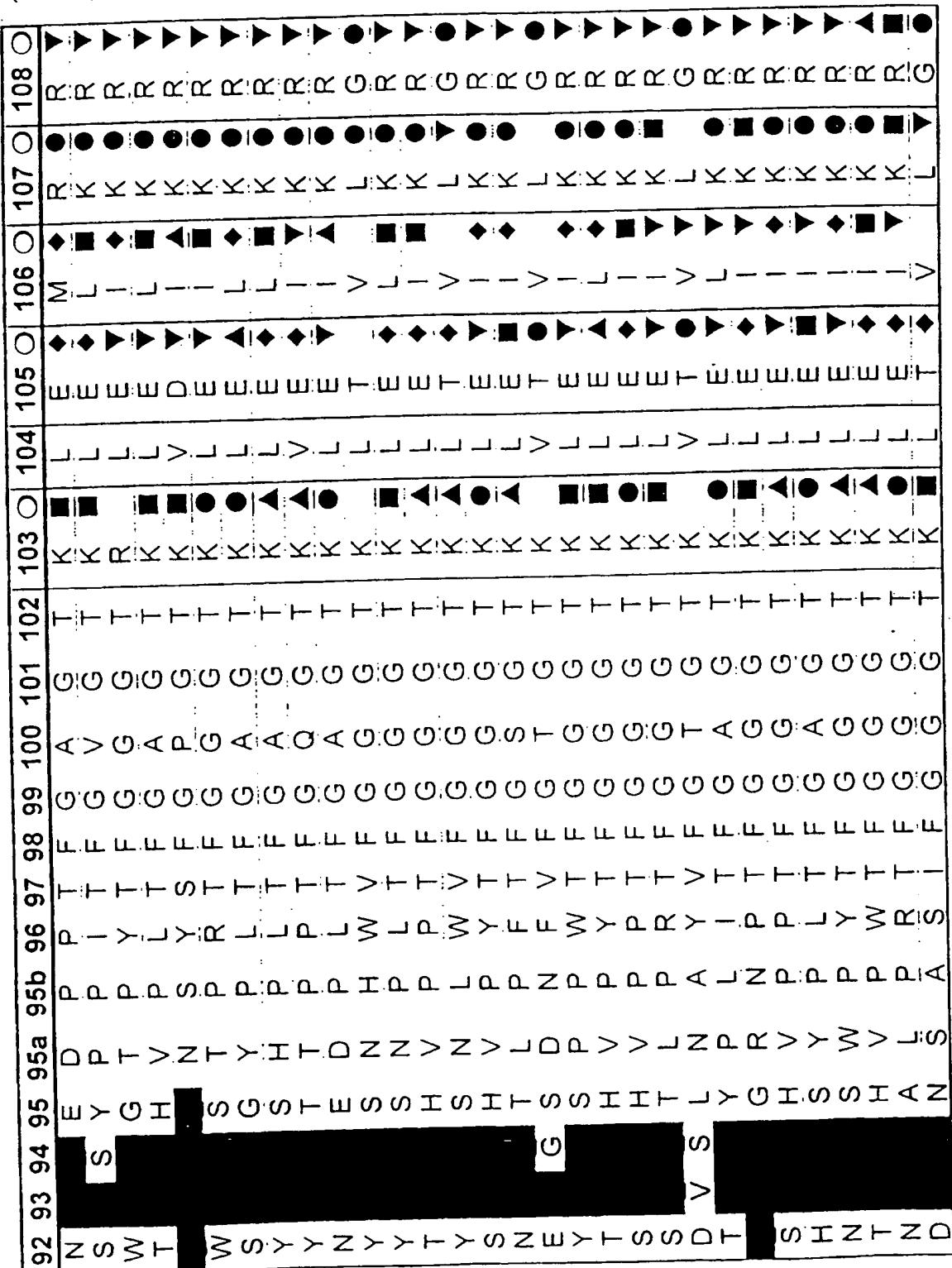


Figure 2b: Variable/constant domain interface residues for VH

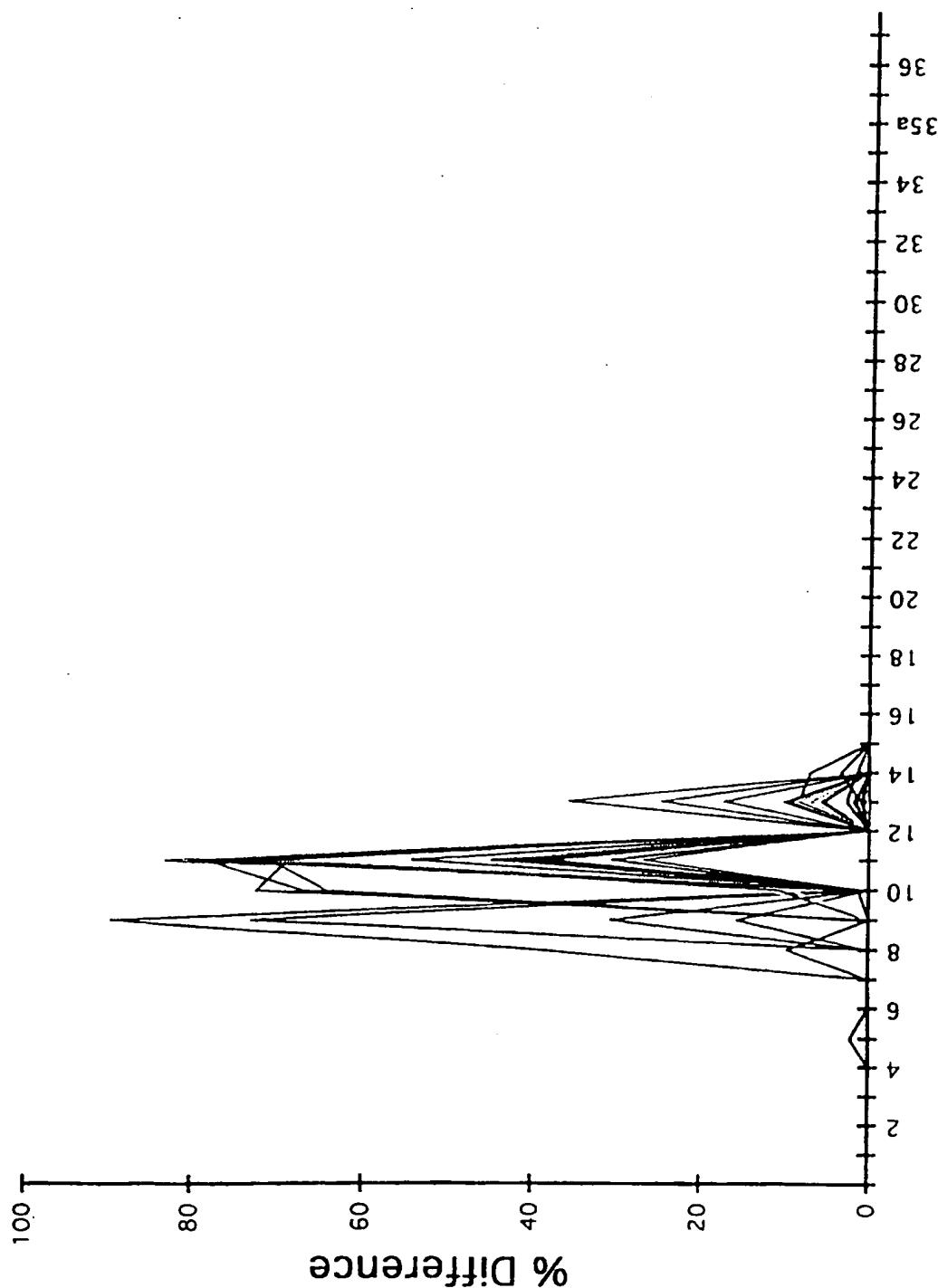
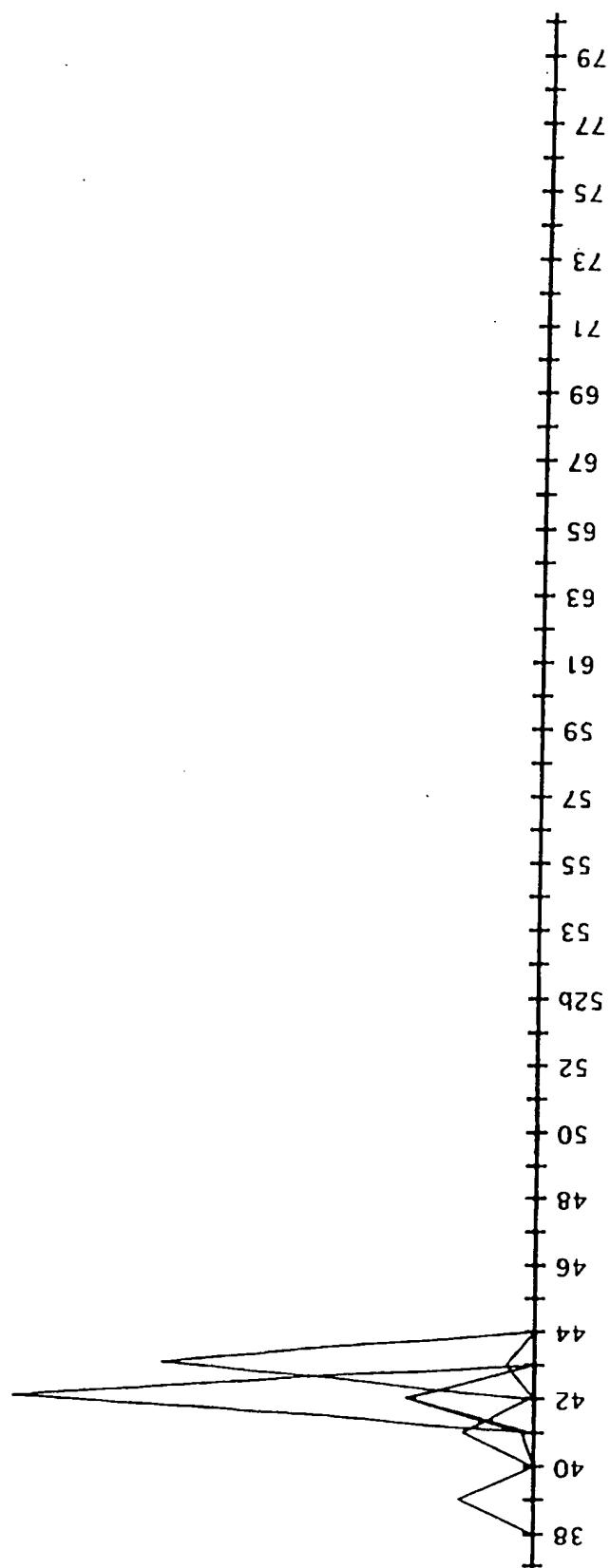
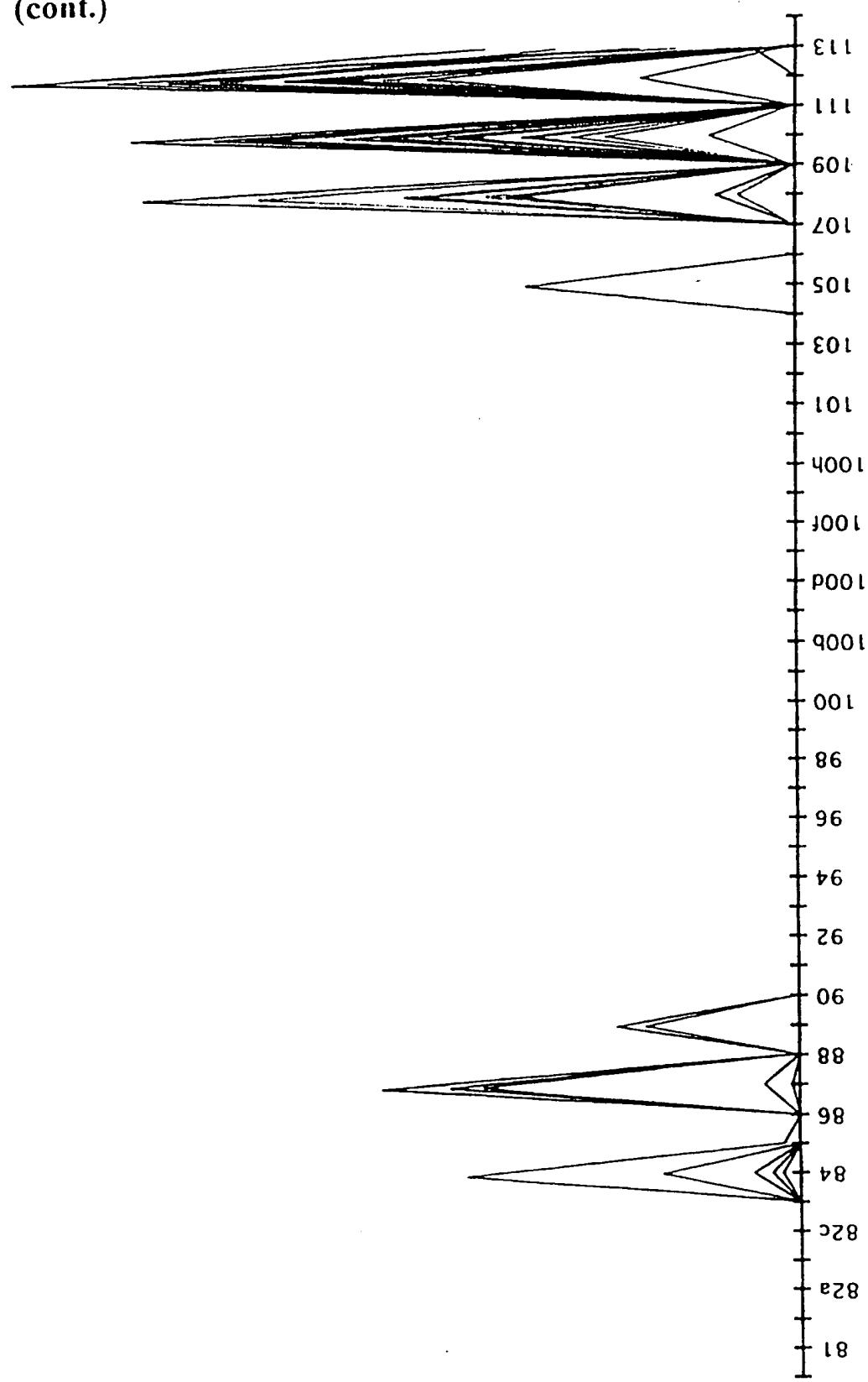


Figure 2b: Variable/constant domain interface residues for VH  
(cont.)



11/27

**Figure 2b: Variable/constant domain interface residues for VII  
(cont.)**



**Figure 2b: Variable/constant domain interface residues for VH (cont.)**

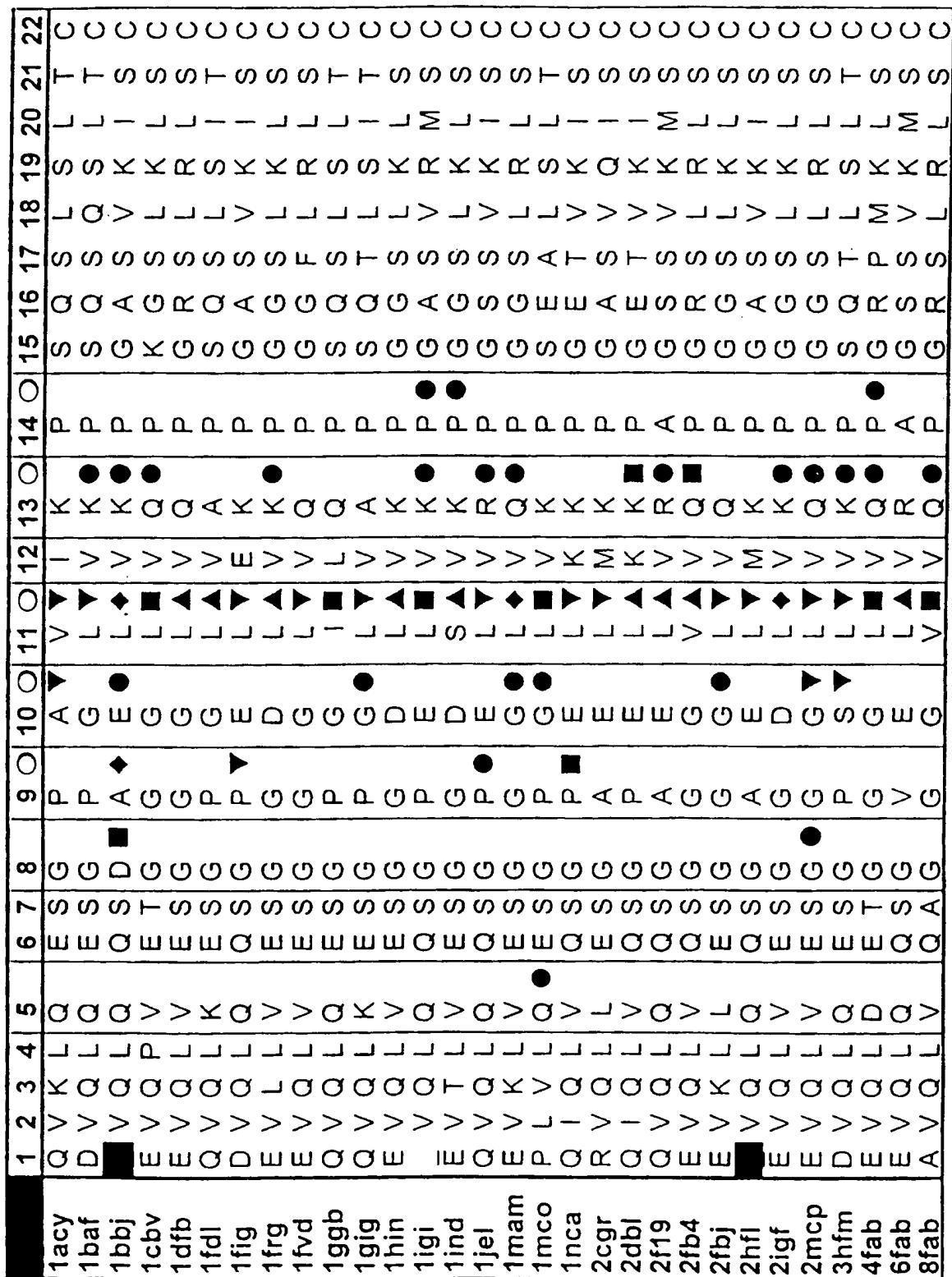
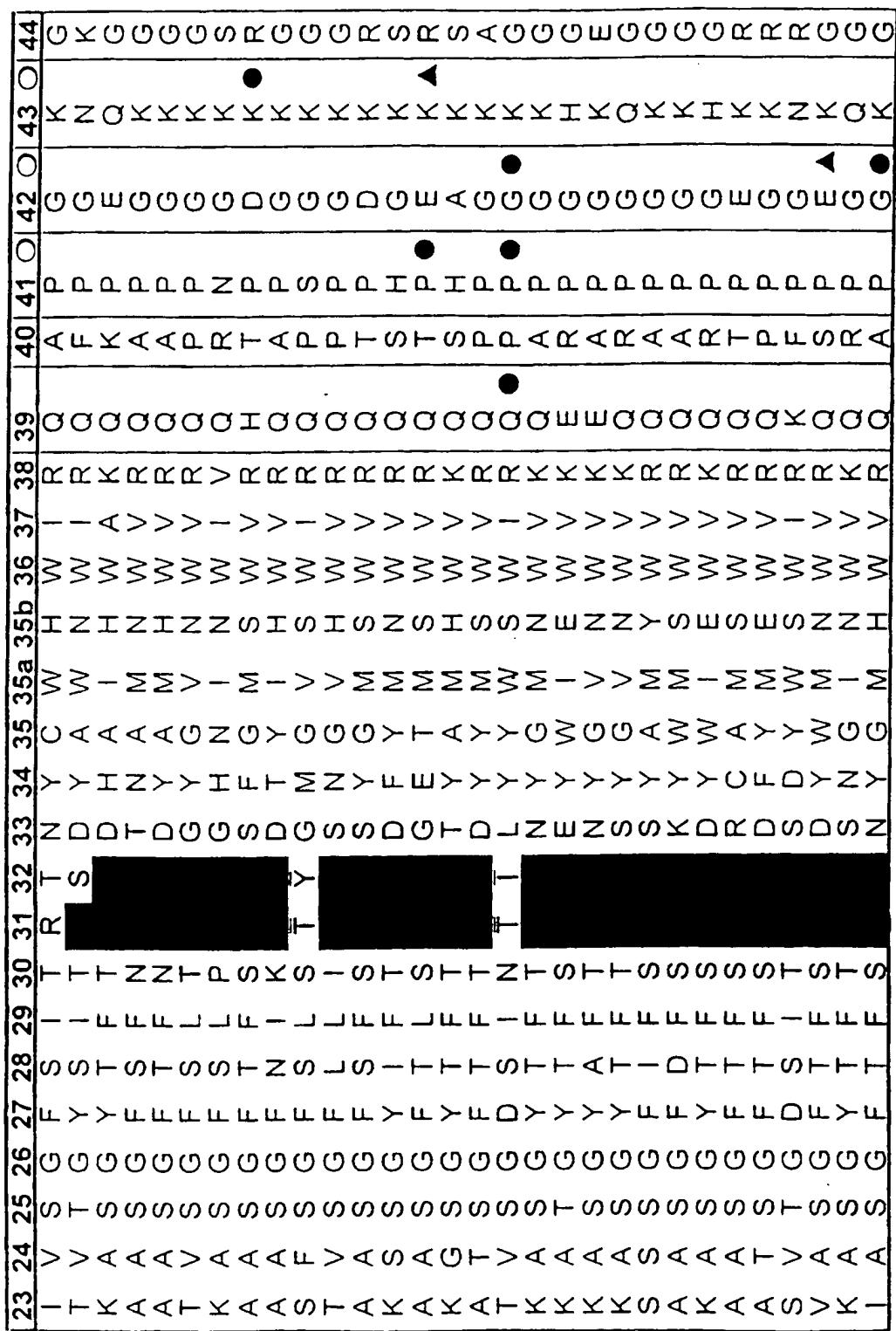


Figure 2b: Variable/constant domain interface residues for VH  
(cont.)

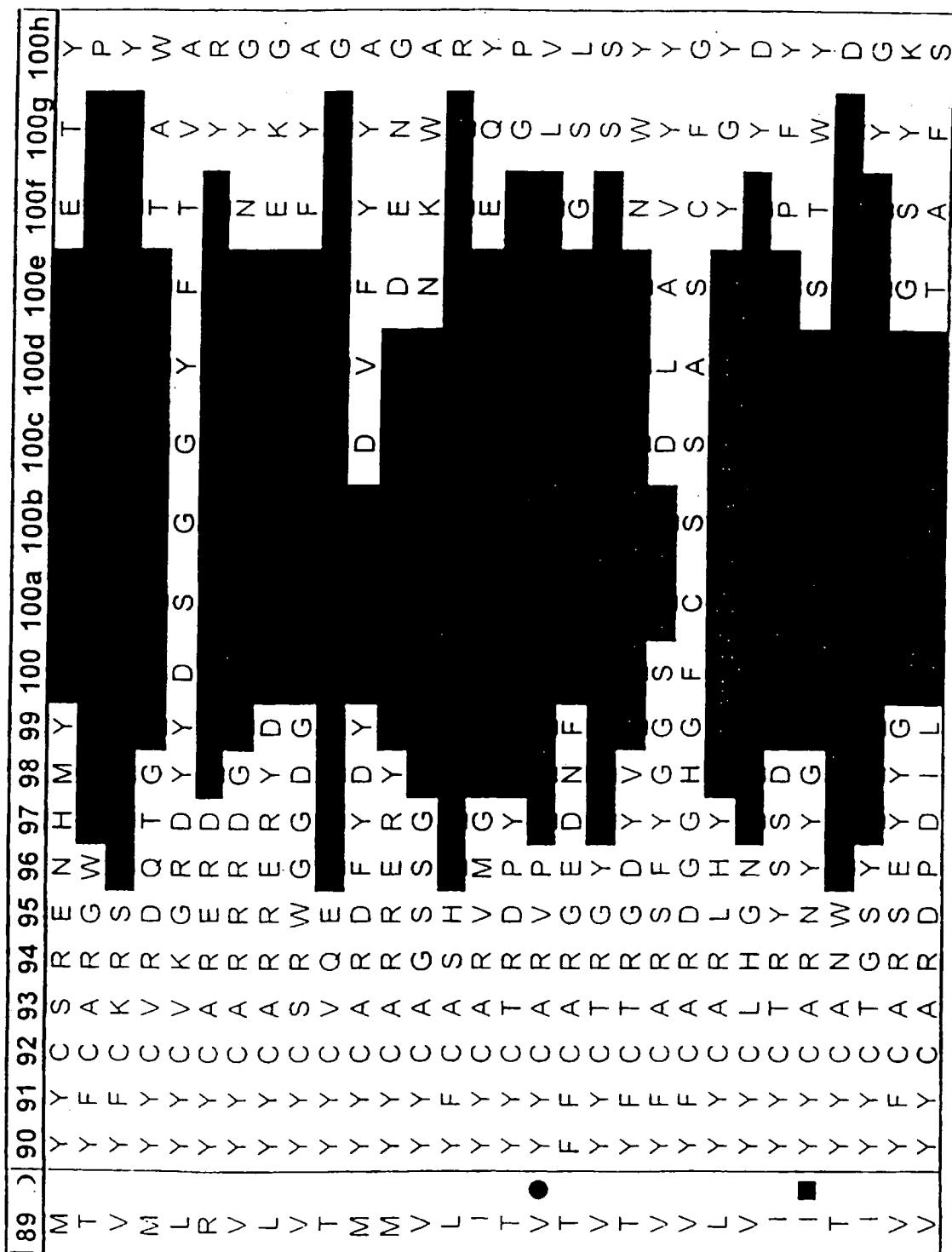


**Figure 2b: Variable/constant domain interface residues for VH (cont.)**

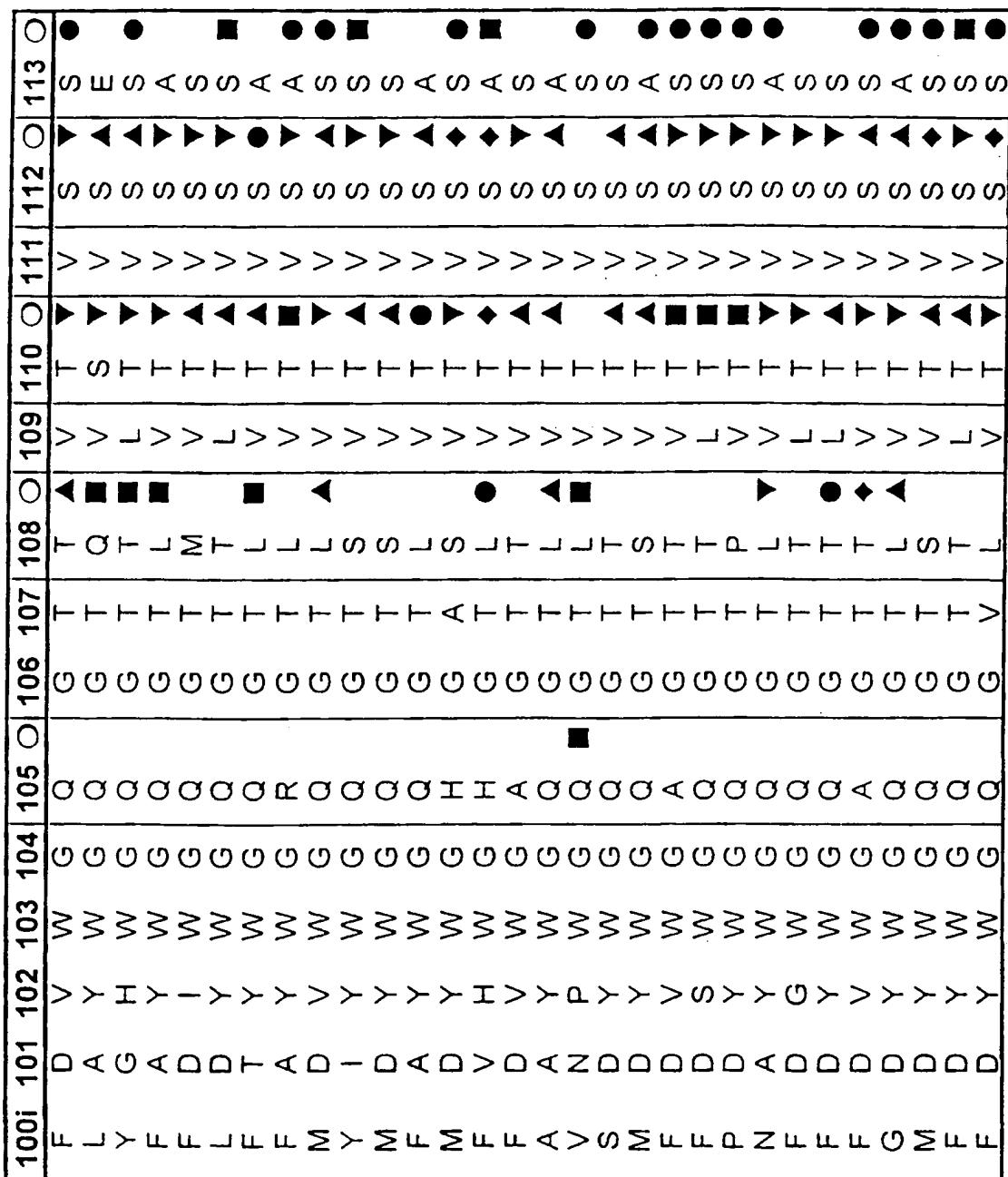
**Figure 2b: Variable/constant domain interface residues for VH  
(cont.)**

68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	82a	82b	82c	83	84	85	86	87	88	
A	A	A	A	A	A	A	A	G	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	
T	T	S	T	M	T	S	A	T	T	S	S	T	S	T	S	T	T	S	T	A	T	S	T	
D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	
E	W	W	W	E	W	W	E	D	W	W	W	W	A	W	W	W	W	W	W	W	W	W	W	E
N	T	S	T	A	T	S	S	S	A	T	S	S	A	Z	S	Z	S	S	A	T	>	S	T	
T	T	K	R	H	O	K	R	D	O	K	T	R	T	R	T	K	T	R	R	T	R	T	R	
V	>	L	L	L	L	L	L	>	L	L	L	L	>	L	L	L	>	L	L	>	L	L	L	
S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	
-	X	Z	Z	Z	Z	T	T	Z	T	X	S	R	Z	A	Z	S	Z	R	D	S	Z	N	Z	
L	L	L	L	L	L	L	L	-	S	S	S	S	-	S	S	S	-	S	S	S	S	S	S	
Q	Q	Q	Q	Q	Q	Q	Q	X	W	Q	X	W	Q	X	W	Q	X	W	Q	Q	Q	Q	Q	
-	L	S	L	L	L	S	S	L	S	L	S	L	S	L	S	L	S	L	S	L	S	L	S	
F	F	Y	Y	Y	Y	F	F	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	
F	F	A	L	L	>	L	L	>	L	L	>	L	A	L	F	A	A	A	A	L	L	>	A	
K	O	T	N	S	O	T	T	T	O	O	T	T	Z	T	-	O	T	T	S	T	T	-	O	T
N	Z	S	N	S	S	Z	Z	S	N	S	S	S	Z	S	N	S	N	S	S	R	S	N	S	
L	K	S	O	K	K	S	X	Z	K	S	O	S	K	A	S	K	X	S	R	K	K	S	K	
S	S	S	A	S	S	A	S	S	A	S	S	A	S	S	A	S	S	A	S	S	A	S	S	
T	T	K	D	O	Z	Z	X	Z	T	T	T	T	R	Z	T	Z	T	D	X	N	T	D		
R	R	A	R	R	K	K	>	R	A	K	K	R	>	R	>	R	>	A	L	>	R	R	R	R
S	T	T	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
T	S	T	T	K	S	T	T	T	T	A	T	T	A	T	T	T	T	T	T	T	T	T	T	

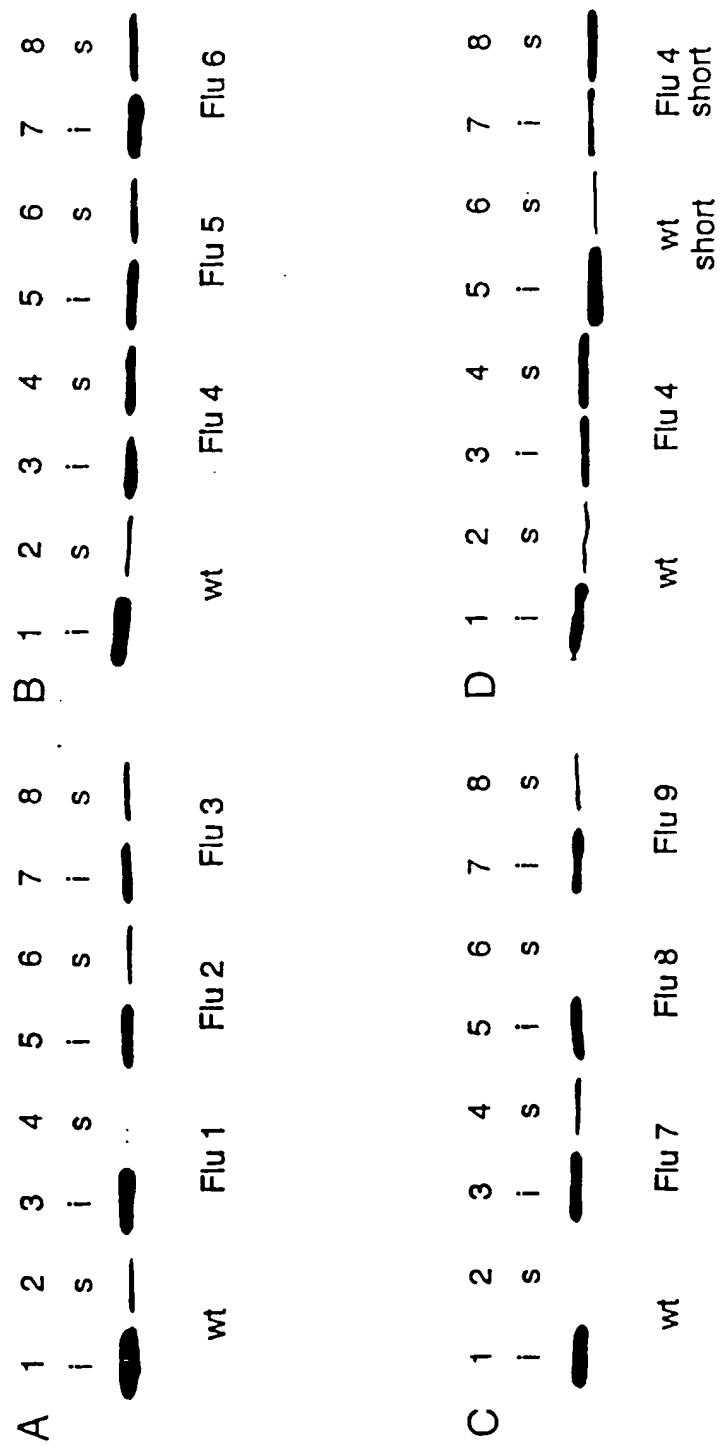
**Figure 2b: Variable/constant domain interface residues for VII (cont.)**



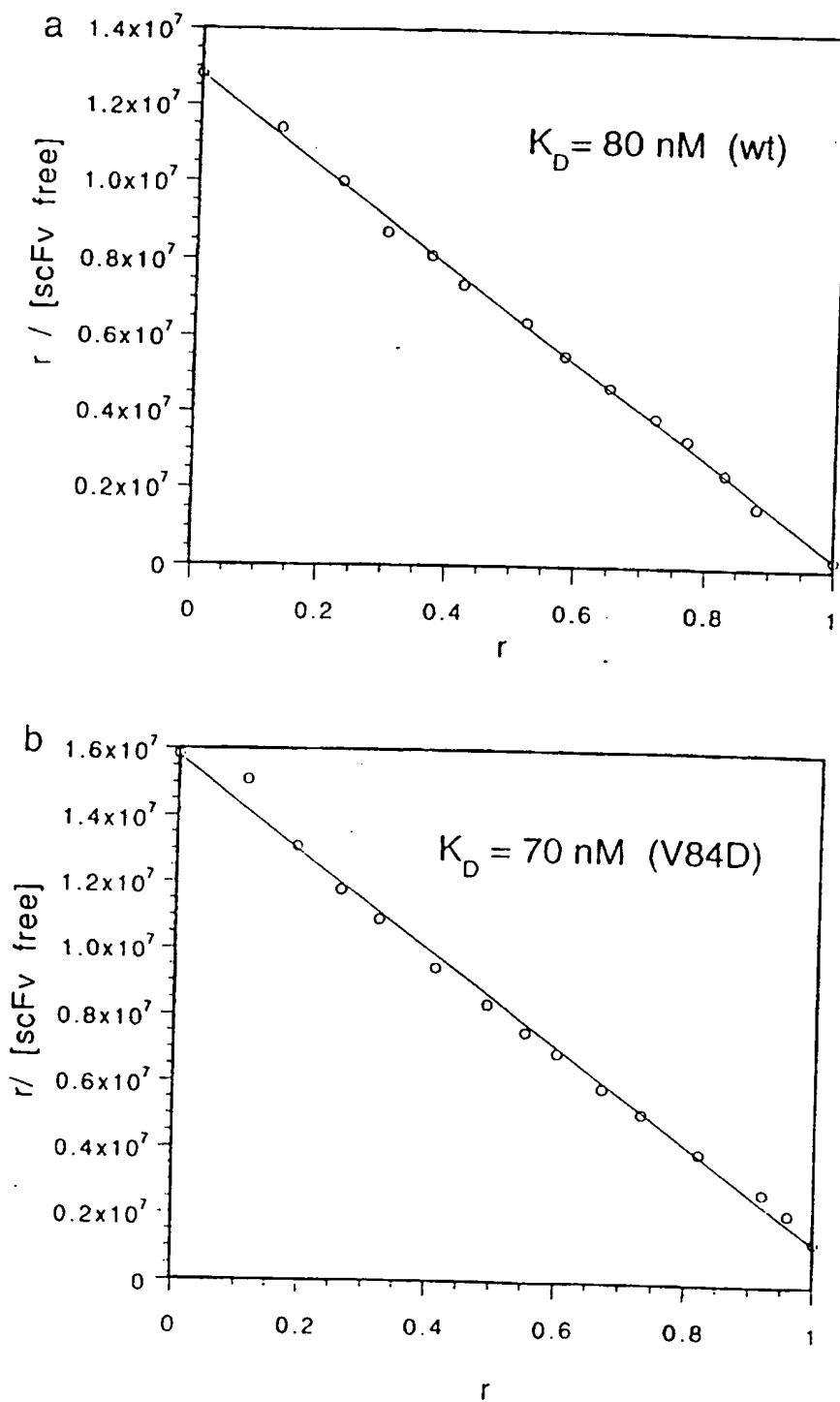
**Figure 2b: Variable/constant domain interface residues for VH (cont.)**



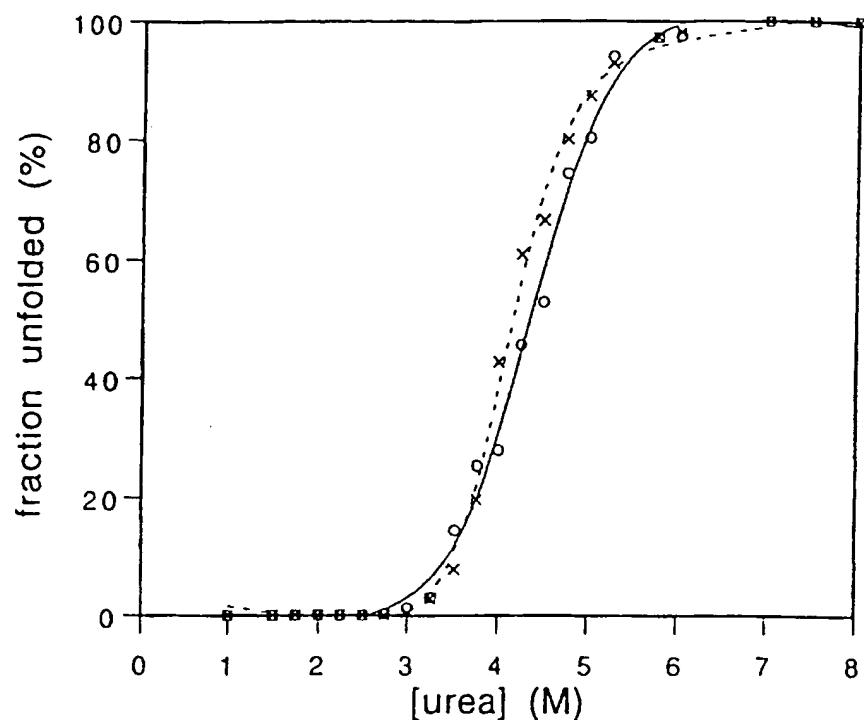
**Figure 3: Western blots showing the insoluble (i) and soluble (s) fractions of cell extracts**



**Figure 4: Scatchard plots of fluorescence titration of fluorescein with antibody: a) Titration of wt scFv;  
b) Titration of Flu4(V84D)**



**Figure 5: Overlay plot of urea denaturation. (x) wt scFv, (o) Flu4**



**Figure 6: Thermal denaturation time courses at 40°C and 44°C for wt and Flu4 scFv fragments**

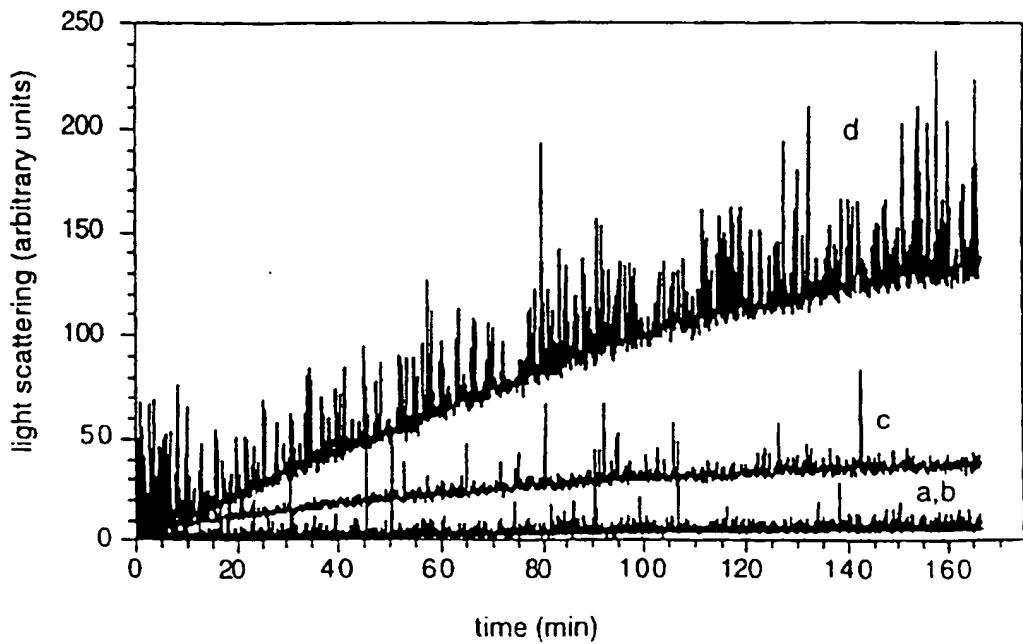


Table 1: Sequence variability of residues contributing to the v/c interface

Position	L9			L10			L12			L15			L39		
	% exp. (FAB)	59	65	10	41	47	12	49	48	0	49	48	37	37	1
Species	kappa	lambda	kappa	kappa	lambda										
Seq. 4-4-20	hu	mu	mu	hu	mu	hu	mu	hu	mu	hu	mu	hu	mu	hu	mu
Consensus	Leu			Ser				Pro		Leu				Lys	
Distribution:	Ser	Ala	Ser	Ala	Thr	Ser	Ser	Ser	Thr	Pro	Leu	Pro	Lys	Lys	Lys
Asp	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Glu	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lys	0	6	0	0	0	0	0	0	0	0	0	0	83	92	30
Arg	0	0	0	0	0	0	0	0	0	0	0	0	14	7	11
His	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Thr	0	4	0	1	49	6	0	3	0	93	1	0	1	0	0
Ser	43	24	90	4	48	60	84	50	99	0	0	0	0	0	0
Asn	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Gln	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Gly	25	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Ala	11	41	7	90	0	0	4	23	0	0	0	8	1	0	0
Cys	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pro	0	2	0	0	0	0	0	11	20	0	0	45	28	84	96
Val	1	0	2	1	0	0	0	0	0	0	0	41	10	0	0
Ile	0	0	0	3	0	24	0	0	0	0	1	2	2	0	0
Leu	11	19	0	0	0	2	0	0	0	0	1	1	49	14	4
Met	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Phe	0	1	0	0	0	2	7	0	0	0	0	1	0	0	3
Tyr	0	0	0	0	0	0	1	0	0	0	0	0	0	2	3
Trp	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

**Table 1: Sequence variability of residues contributing to the v/c interface (cont.)**

**Table 1: Sequence variability of residues contributing to the v/c interface (cont.)**

Table 1: Sequence variability of residues contributing to the v/c interface (cont.)

Position	H9	H10	H11	H13	H14	H41	H42	H43
% exp. (FAB)	29	65	31	69	36	72	111	65
% exp. (ind.)	33	72	71	73	36	72	115	78
% buried v/c	7	8	58	5	0	0	3	2
Species	hu	mu	hu	mu	hu	mu	hu	mu
Seq. 4-4-20	Gly	Gly	Leu	Gln	Pro	Pro	Glu	Lys
Consensus	Gly	Pro	Gly	Glu	Leu	Lys	Pro	Gly
Distribution:	Asp	0	0	5	2	0	0	0
	Glu	2	0	27	54	0	0	2
	Lys	0	0	0	0	59	54	0
	Arg	0	0	1	0	3	0	19
	His	0	0	0	0	0	0	0
	Thr	1	2	1	0	0	0	0
	Ser	1	1	0	2	0	0	0
	Asn	0	0	0	0	0	0	0
	Gln	0	0	0	0	0	0	0
	Gly	42	29	61	38	0	0	0
	Ala	33	31	3	1	0	0	0
	Cys	0	0	0	0	0	0	0
	Pro	21	36	0	0	0	96	85
	Val	0	1	0	1	38	1	1
	Ile	0	0	0	0	0	0	0
	Leu	0	0	0	0	0	0	0
	Met	0	0	0	0	0	0	0
	Phe	0	0	0	0	0	0	0
	Tyr	0	0	0	0	0	0	0
	Trp	0	0	0	0	0	0	0

**Table 1: Sequence variability of residues contributing to the v/c interface (cont.)**

**Table 2: Mutations introduced in the scFv fragment of the antibody 4-4-20**

	L15E (V <sub>D</sub> )	L11N (V <sub>H</sub> )	L11D (V <sub>H</sub> )	V84D (V <sub>H</sub> )
Flu 1	•			
Flu 2		•		
Flu 3			•	
Flu 4				•
Flu 5		•		•
Flu 6			•	•
Flu 7	•	•		
Flu 8	•		•	
Flu 9	•	•		•
Flu 4 short				•

**Table 3: K<sub>D</sub> values of the different scFv mutants determined in fluorescence titration**

	Flu wt	Flu 3	Flu 4	Flu 6	Flu wt#
K <sub>D</sub> (nM)	80 ± 7	60 ± 12	70 ± 10	75 ± 13	90

# INTERNATIONAL SEARCH REPORT

In. national Application No

PCT/EP 97/03792

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC 6	C07K16/00	C12N15/12	C12N15/13	C12N15/63	G01N33/53
	A61K38/17		A61K39/395		

According to International Patent Classification(IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K C12N G01N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 92 01787 A (CAMBRIDGE ANTIBODY TECH) 6 February 1992 cited in the application see whole document, esp. page 3 ff.	1-16, 20, 25-34
Y	HOPP T. P. ET AL.,: "A short polypeptide marker sequence useful for recombinant protein identification and purification" BIO/TECHNOLOGY, vol. 6, - October 1988 pages 1204-1210, XP002046115 cited in the application see the whole document	21, 22
	---	21, 22
	---	---
	---	---

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

\* Special categories of cited documents :

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- \*&\* document member of the same patent family

2

Date of the actual completion of the international search

7 November 1997

Date of mailing of the international search report

24/11/1997

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 851 epo nl.  
Fax: (+31-70) 340-3016

Authorized officer

Müller, F

**INTERNATIONAL SEARCH REPORT**

International Application No  
PCT/EP 97/03792

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MUYLDERMANS S ET AL: "SEQUENCE AND STRUCTURE OF VH DOMAIN FROM NATURALLY OCCURRING CAMEL HEAVY CHAIN IMMUNOGLOBULINS LACKING LIGHT CHAINS" PROTEIN ENGINEERING, vol. 7, no. 9, 1 September 1994, pages 1129-1135, XP000445081 see abstract and p1130, 2.column, 7.para. -p1132	1-12
Y	JENKINS T. M. ET AL.: "Catalytic domain of human immunodeficiency virus type 1 integrase: Identification of a soluble mutant by systematic replacement of hydrophobic residues" PROC. NATL. ACAD. SCI. USA, vol. 92, - June 1995 pages 6057-6061, XP002046116 see abstract and discussion	13,18, 19,26-34
Y	BREITLING F. ET AL: "A surface expression vector for antibody screening" GENE, vol. 104, - 1991 pages 147-153, XP002046118 see the whole document	13,18, 19,26-34
A	DALE J.E. ET AL.: "Improving protein solubility through rationally designed amino acid replacements: solubilization of the trimethoprim resistant type S1 dihydrofolate reductase" PROTEIN ENGINEERING, vol. 7, no. 7, - 1994 pages 933-939, XP002046117 see abstract, discussion p938, column 1; 1. paragraph	17
A		1-34

**INTERNATIONAL SEARCH REPORT**

## Information on patent family members

International Application No

PCT/EP 97/03792

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9201787 A	06-02-92	AU 8238291 A CA 2087095 A EP 0540586 A JP 6502526 T	18-02-92 26-01-92 12-05-93 24-03-94

**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

**BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- BLACK BORDERS**
- IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- FADED TEXT OR DRAWING**
- BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- SKEWED/SLANTED IMAGES**
- COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- GRAY SCALE DOCUMENTS**
- LINES OR MARKS ON ORIGINAL DOCUMENT**
- REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- OTHER:** \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**